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DL-FORMING LACTIC ACID BACTERIA

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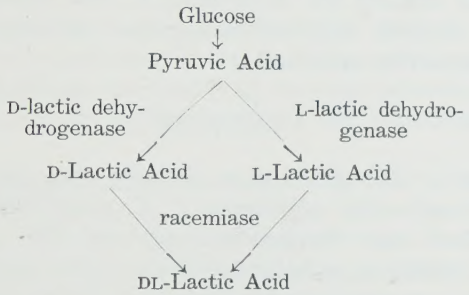
INTRODUCTION

Lactic acid bacteria may be unique among living organisms in that they can produce various kinds of lactic acid, the optical form of which is characteristic for different species.

DL-Lactic acid is formed from sugar via pyruvic acid which is first transformed to D- or L-lactic acid by the action of the corresponding lactic dehydrogenase. Racemization by a specific enzyme racemiase, lactic racemase, follows.

Scheme 1. Pathways from glucose to DL-lactic acid[1].



Accordingly, lactic acid bacteria can be classified into two groups, namely D- and L-formers, and each of these can be further subdivided on the basis of presence or absence of racemiase (see Table 1).

Table 1. A tentative classification of lactic acid bacteria.

Lactic acid bacteria	D-formers	racemiase −	D-formers	Group I
		racemiase +	D+DL~DL-formers	" II
	L-formers	racemiase +	DL~DL+L-formers	" III
		racemiase −	L-formers	" IV

When racemiase is present but its activity is not sufficient, the D-formers will produce DL+D-lactic acid whereas the L-formers will produce DL+L-acid; thus these bacteria belong to subgroups II and III respectively. But when the enzyme is fairly active the lactic acid appears in an entirely racemized form, so we can't determine whether the test strain is a D- or L-former, that is through which path of Scheme 1 glucose will be changed into DL-lactic acid.

Since lactic acid bacteria generally contain both D- and L-lactic dehydrogenases even if they produce active lactic acid[2], we cannot use the presence of these enzymes for classifying the bacteria into D- or L-formers. For example, *Lactobacillus bulgaricus* 223 contains both types of lactic dehydrogenase and although the D-enzyme is far more active than the L-enzyme, still this strain produces L-lactic acid.

We found that racemiase requires diphosphopyridinenucleotide (DPN) as coenzyme and that the dissociation constant between the enzyme and the coenzyme was $1.2\times10^{-4}M$[3]. This value is markedly larger than the known value of $5\times10^{-6}M$ for lactic dehydrogenase and DPN. It means that DPN combines more closely with lactic dehydrogenase than with racemiase. Consequently, if DPN formation were suppressed step by step, racemiase activity would be inhibited before any restriction of lacticdehydrogenase occured. A certain amount of active lactic acid may be found in the product thus formed.

To further study this process the present authors examined the nutritional dependency of DL-lactic acid formers on nicotinic acid and adenine, which can be considered as precursors of DPN. As nicotinic acid is a common essential nutrient for DL-formers, it was possible, by restricting the supply of nicotinic acid, to make these bacteria to form greater or lesser amounts of active lactic acid.

METHODS

Strains used : — Six strains of DL-formers were used as test organisms. Five of them, *Lactobacillus plantarum* 11, *L. plantarum* 256, *L. arabinosus* 17–5, *L. japonicus*, and *Pediococcus hennebergi* 253, are homofermenters while *L. brevis* X4221 is a heterofermenter. For comparison L(*dextro*)-lactic acid forming homofermenters such as *L. bulgaricus* 223, *L. casei* 372, and an undetermined strain N 18, and the heterofermentative D(*laevo*)-former, *L. batatas*[4], were also used. All strains except *L. arabinosus* were authenticated in our own laboratory[5]. We had wanted to use *L. sake* as one of DL-formers, but since no strain of this species could grow in the semi-synthetic basal medium described below, we were obliged to give it up this time.

Basal medium : — The following semi-synthetic basal medium was used throughout the experiments.

One liter of medium contains: glucose 20 g, Na-acetate 20 g, casein-(HCl)-hydrolysate 10 g, DL-tryptophan 0.1 g, L-cystine 0.1 g; [*vitamins*] thiamin 1.0 mg, riboflavin 1.0 mg, pyridoxine 1.0 mg, pyridoxal 0.2 mg, Ca-pantothenate 1.0 mg, biotin 10 μg, folic acid 10 μg, nicotinic acid 1.0 mg: [*bases*] adenine-H_2SO_4 10 mg, guanine-HCl 10 mg, xanthine 10 mg, uracil 10 mg; [*inorganic salts*] solution A 10 ml, solution B 10 ml.

Salt solution A contains: KH_2PO 25 g, K_2HPO_4 25 g in 250 ml. water.

Salt solution B contains: $MgSO_47H_2O$ 10 g, NaCl 0.5 g, $FeSO_47H_2O$ 0.5g, $MnSO_4$-$4H_2O$ 0.5 g in 250 ml. water.

Bacterial growth : — As we know well, growth is not the same as fermentation. But since lactic acid formation takes place in parallel with growth within certain limits, growth rates were compared by measuring alkali titration values under suitable conditions as in the ordinary procedures of microbiological assay.

Optical properties of lactic acid : — For lactic acid, the notations D- and L- are reversed from the ordinal meanings of *dextro-* and *leavo-* rotatory ; D-lactic acid corresponds with the old *leavo-* or *l*-lactic acid and L-acid corresponds with the former *dextro*-lactic acid.

For determinations a micromanometic method using bacterial D- or L-lactic dehydrogenases, proposed by Kitahara and Fukui[6], was used in addition to the usual Zn-salt method.

If 100 μg of a sample of 250 μg were determined as D-lactic acid, the residual 150 μg must be L-lactic acid. Thus the constitution of this sample is determined to be 40%D+60%L, or 80%DL+20%L. In this paper it will be referred to describe as DL+L (20%).

EXPERIMENTAL RESULTS

(1) *Requirements for nicotinic acid and organic bases.*

The requirements of the test organisms for nicotinic acid and organic bases were examined by eliminating the requisite substances from the basal medium. Table 2 shows the results of the first experiments ; it indicates that nicotinic acid is essential for all of the test organisms, but adenine is not.

It might be thought that the reason adenine is not needed in the medium is that other purine and pyrimidine bases are transformed into adenine ; however, the experimental data of Table 3 indicate that most strains can grow without any sort of purine or pyrimidine bases. In other words, these strains can freely synthesize adenine from substances other

Table 2. Requirements for nicotinic acid and adenine.
Culture: 3 days at 30°C.

Strain / Medium	ml of n/10 NaOH per 2 ml. of medium								
	L. plantarum 11	*L. plantarum* 256	*L. arabinosus* 17–5	*L. brevis* X4221	*P. hennebergi* 253	*L. casei* 372	*L. bulgaricus* 223	*L. batatas*	N18
Basal	3.81	3.74	4.52	2.64	2.95	1.45	1.50	2.35	1.25
Basal minus nicotinic acid	0.07	0.14	0.17	0	0.35	0.24	0.10	0.20	0.25
Basal minus adenine	3.82	3.85	4.52	2.65	3.00	1.75	2.00	2.35	0.95
Basal minus nicotinic & adenine	0.16	0.17	0.10	0	0.30	0.25	0.20	0.10	0.20

than purine or pyrimidine bases. It may be of some value to note that uracil and one of the purines are known to be essential for the growth of the undetermined strain, N 18.

Table 3. Requirements for organic bases.
Culture: 3 days at 30°C.

Strain / Medium	ml. of n/10 NaOH per 2 ml. of medium						
	L. plantarum 11	*L. plantarum* 256	*L. arabinosus* 17–5	*L. brevis* X4221	*P. hennebergi* 253	*L. batatas*	N 18
Basal	3.35	3.50	3.55	1.95	2.70	2.10	1.90
Basal −A	3.40	3.40	3.45	1.95	2.55	2.10	1.65
Basal −G, X	3.45	3.50	3.50	1.95	3.00	2.10	1.45
Basal −A, G, X	3.40	3.50	3.65	1.80	2.55	2.10	0
Basal −A, U	3.30	3.45	3.50	1.90	2.55	2.10	0
Basal −G, X, U	3.35	3.50	3.55	1.90	3.10	2.00	0
Basal −A, G, X, U	3.45	3.60	3.60	1.95	2.95	2.05	0
Basal −A, G, X, U, N	0.15	0.10	0.15	0.15	0.15	0.10	0

A: adenine, G: guanine, X: xanthine, U: uracil, N: nicotinic acid.

(2) *Response to nicotinic acid.*

As all DL-forming strains need nicotinic acid for growth, DPN formation may be restricted by limiting the amount of nicotinic acid. Experiments

Table 4. Relationship between nicotinic acid concentration and lactic acid formation.
Culture: 3 days at 30°C.

Nicotinic acid μg/ml. / Strain	ml. of n/10 NaOH per 2 ml. of medium									
	L. plantarum 11	*L. plantarum* 256	*L. arabinosus* 17–5	*L. japonicus*	*L. brevis* X4221	*P. hennebergi* 253	*L. casei*	*L. bulgaricus* 223	*L. batatas*	N 18
1.0 (Basal)	3.10	3.20	3.90	4.00	2.35	3.30	1.70	1.45	2.40	1.00
0.2	2.50	2.65	3.15	3.50	1.95	2.70	1.60	1.55	1.25	1.00
0.1	1.85	2.00	2.45	2.92	0.95	2.40	1.55	1.45	0.85	0.65
0.02	0.75	0.85	0.75	1.25	0.30	1.20	1.05	0.85	0.25	0.25
0	0.25	0.10	0.10	0.20	0	0.20	0.25	0.20	0.15	0.15

were carried out to determine the responses of DL-formers to varying concentrations of nicotinic acid and the results obtained are shown in Table 4.

Lactic acid formation is increased in proportion to the increase in nicotinic acid concentration. In the cases of *L. casei* and *L. bulgaricus*, maximum quantities of lactic acid were attained at a concentration of nicotinic acid of 0.1 μg/ml., but DL-formers in general could not attain their maximum values even when the concentration of nicotinic acid was increased to 0.2 μg/ml. We can not conclude from these results, however, that the maximum growth of active lactic acid formers is achieved in the presence of comparatively small amounts of nicotinic acid. It is more likely that their maximum growth is controlled by some other factor, as was shown by Kennedy *et al*[7].

(3) *The optical properties of lactic acid and the racemiase activity of cells formed in nicotinic acid-restricted medium.*

In the preceding experiments it has been shown that the DL-formers demand nicotinic acid as an essential growth factor, and that their fermenting tendency is increased in paralell with the nicotinic acid concentration within a range of 0~1.0 μg/ml. The formation of DPN appears to be affected considerably by the dosage of nicotinic acid, and experiments were carried out to test this suggestion further.

After the DL-formers were cultured in the nicotinic acid-restricted media, the optical properties of the lactic acid formed and the racemiase activity of the cells were determined.

Racemiase activity: (see Tables 5 and 6) Cells cultured in 60 ml. of medium were centrifuged and thoroughly washed. One half of the wet cells alone, and the other half in combination with 200μg of DPN, were mixed with 9 mg of L-lactic acid

Table 5. Optical activity of lactic acid produced in nicotinic acid-restricted medium, and racemiase activity of the cells.
Culture medium: Adenine was eliminated from basal medium and nicotinic acid was restricted to 0.15μg/ml. Culture: 60 ml, 30°C, 48 hours.

		L. plantarum 11	*L. plantarum* 256	*L. arabinosus*	*L. japonicus*	*L. brevis*	*P. hennebergi*
Glucose (mg)	initial	1,500	1,500	1,500	1,200	1,500	1,500
	consumed	1,500	1,300	1,300	695	970	1,500
Lactic acid	obtained (mg)	1,280	1,040	900	580	430	1,200
	optical property	DL+D(45%)	DL+D(50%)	DL+D(56%)	DL+D(47%)	DL	DL+L(91%)
Racemiase activity (μg)	cells alone	280	180	260	300	175	0
	cells +DPN	530	310	880	540	350	0

(Na-salt). The racemized moiety (μg) was determined by the above-mentioned micro-manometric method after incubating for 4 hours at 30°C. In the present experiments this method has been used only for comparing the activities of cells with and without DPN.

Table 5 shows that when the concentration of nicotinic acid is brought down to 0.15 μg/ml., every strain except *L. brevis* produces lactic acid with measurable optical activity.

Table 6. Effect of nicotinic acid concentration upon the optical activity of lactic acid.

Culture medium: Addition of bases and concentration of nicotinic acid in basal medium were modified as indicated. Culture: 60 ml., 30°C

Strain		*L. plantarum* 11		*P. hennebergi*	*L. brevis*
Bases		none	adenine eliminated	all	none
Nicotinic acid		0.05μg/ml.	1.0μg/ml.	2.0μg/ml.	0.1μg/ml.
Incubation time (hours)		48	48	48	24
Glucose (mg)	initial	1.800	1.500	1.820	1.740
	consumed	720	1.500	1.350	520
Lactic acid	obtained (mg)	500	1.250	1.190	203
	optical form	DL+D (40%)	DL	DL+L (38%)	DL+D (10%)
Racemiase activity (μg)	cells alone	not detectable	840	470	not detectable
	cells +DPN	,,	920	560	,,

Compare with the data of corresponding strains in Table 5.

In the case of *L. plantarum* 11, if the level of nicotinic acid is further suppressed to 0.05 μg/ml, lactic acid formation falls but the proportion of the active fraction is not increased. However, if nicotinic acid is supplied at the standard level, *i.e.* 1.0μg ml., the resulting lactic acid is, as under ordinary culture conditions, absolutely inactive. (See Table 6)

In *L. brevis* the racemiase formation probably be perfomed so regularly, that the active part of lactic acid can be identified only when the nicotinic acid level and the culture-time are extremely restricted.

On the other hand, we failed to make *P. hennebergi* produce entirely recemized lactic acid even when the supply of nicotinic acid was made twice the standard concentration.*

* According to the original description[8], this strain formed DL+L (14%) -lactic acid in koji-boillon medium.

DISCUSSION

Nicotinic acid is essential for growth of, and subsequent fermentation by DL-forming lactic acid bacteria, while purine and pyrimidine bases are not.

Nicotinic acid is considered as a precursor of DPN, so the reason the DL-formers produce more optically active lactic acid than normal when the supply of nicotinic acid is restricted is that recemiase activity is suppressed owing to insufficient formation of the coenzyme, DPN.

This is again concluded from the fact that the racemiase activity of the cells runs parallel to the nicotinic acid concentration in the culture medium, and the activity of nicotinic acid-restricted cells is reinforced by adding DPN.

This phenomenon is quite similar to the facts reported by Gunsalus and his co-workers[9], concerning to the amino acid decarboxylase and the trans-aminase of *Streptococcus faecalis* ; in cells taken from vitamin B_6-restricted medium these enzymes are activated by adding pyridoxal phosphate, the coenzyme derived from vitamin B_6.

The racemiase of *L. plantarum* and *L. arabinosus* is like the above-mentioned enzymes of *S. faecalis*, though the apoenzyme is suffcient, the enzyme can not exhibit full activity owing to a deficiency of its coenzyme.

In *P. hennebergi*, since the racemiase could not be activated by the supply of DPN, the synthesis of apoenzyme in this species appears weak.

It appears worth-while from the view-point of natural systematics that DL-lactic acid formers should be classified into D- and L-formers according to whether they pass through the left or right paths of Scheme 1.

In Bergey's Manual[10], *L. arabinosus* Fred et Peterson is appropriately described as a synonym of *L. plantarum* Orla-Jensen from the general systematical point of view. Our present experiments confirm this opinion.

L. japonicus Kitahara et Ishida[11] is said to be a DL-forming, mesophilic, active fermenter of plant origin, and may be differentiated from *L. plantarum* by the absence of the ability to ferment pentoses and lactose. The present experiments lead to the conclusion that this species may be appropriately considered a racemiase positive *L. leichmannii*.[12]*

It is interest to note that not only *L. japonicus*, but also *L. plantarum* and *L. arabinosus*, fall into a group of the same category as *L. leichmannii* and *L. delbrückii*, all of which decompose sugar through the left path of Scheme 1.

Kopeloff and Kopeloff[13] found that the so-called S-forms of *L. acidophilus* produced L-lactic acid, whereas the R-forms produced DL- or L-lactic acid, differing according to the age of culture. This proves that *L. acidophilus* is different from the D-forming *L. plantarum* but rather similar to

* *L. leichmannii* is a mesophilic D-former which can not ferment either pentoses or lactose.

the L-forming *L. bulgaricus* and *L. casei*.

Katagiri and Kitahara[14] showed that *L. sake* produced DL-, DL+L, or L-lactic acids, differing according to strains and experimental conditions. In Bergey's Manual this species is described as similar to *L. plantarum*; we prefer the idea that it is rather closer to Kopeloffs' *L. acidophilus* from the view-point of the optical properties of lactic acid.

In 1940 one of the present authors[5] differentiated the genus Pediococcus from Streptococcus principally on the basis of the existence of racemiase, though they differ also in morphological characteristics. L-Forming Streptococcus and DL+L-forming Pediococcus must have a close relationship to one another.

Because of the racemiase formation of the heterofermenting *L. brevis* took place relatively more easily than the lactic acid formation, we could only demonstrate the formation of optically active D-lactic acid by imposing extremely limited conditions. However, it may be of interest to note that this species is related not only to the heterofermenting, racemiase negative, rod-shaped *L. batatas* but also to the heterofermenting coccus, Leuconostoc, and to the rather complex fermenting bacteria such as Escherichia and Aerobacter.

SUMMARY

(1) All the DL-forming lactic acid bacteria used in this research, i.e., *Lactobacillus plantarum* (2 strains), *L. arabinosus*, *L. japonicus*, *L. brevis*, and *Pediococcus hennebergi*, demand nicotinic acid as an essential growth factor, while organic bases such as purines and pyrimidines are not essential for these strains.

(2) If DL-formers are cultured in a medium which is limited in nicotinic acid, the resulting lactic acid reveals measurable optical activity. This is caused by the suppression of the formation of co-racemiase, DPN, by the insufficiency of its precursor.

(3) By determining the optical forms of lactic acid thus formed, DL-formers can be divided into D- and L-fomers. This fact is considered fundamental from the standpoint of natural systematics.

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HEREDITARY VARIATION AND GENETIC RECOMBINATION IN KOJI-MOLDS (*ASPERGILLUS ORYZAE* AND *ASP. SOJAE*)

I. NATURAL VARIATION

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INTRODUCTION

Koji-molds (*Aspergillus oryzae* and *Asp. sojae*) are widely used for sake, soybean paste, soy sauce and other fermentation products, and they have an extensive distribution in Japan. They have long been known to be very liable to variation during preservation as stock cultures, and to give rise spontaneously to sectors or overgrowths, in pure cultured colonies. Although considerable attention has been paid to such variation in the course of making "Tane Koji" which is used as a starter for Koji-making in the fermentation industries, no systematic experiments have yet been made to determine the cause of this variation.

In 1949 the present investigations were initiated to study whether or not there is any difference in variability among strains of Koji molds, the tendency and extent of variation, and the mechanism of variation.

In the field of practical application, the production of a good yield of enzyme activity (amylase and protease) in fermentation cultures has also been a matter of economic importance. In all these fermentations, the improvement of the strains so far has been based on selection either among large collections of strains from nature or factories, or from spontaneous mutations in one strain. Studies on the improvement of Koji-mold strains by means of induced mutation have recently been done by Iguchi[1-3], and he[4] succeeded in producing enzymes with high potencies. This work has been continued in our laboratory. One of the methods used is to make mixed cultures to obtain heterocaryosis — expecting to reap the benefits of heterocaryotic vigor (Dodge[5]; Beadle and Coonradt[6]) or of combination of high enzyme activities of different strains. Recently our work[7] demonstrated the possibility of the production of new types of strains which are recombinants associating two or more desirable properties present separately in different strains or species. It was posturated by Pontecorvo and co-workers[8] that the recombinants or heterozygous diploid strains are achieved by a "para-sexual" mechanism.

Koji-molds are not always the most suitable material for genetic in-

vestigations since they are asexual fungi and form multinucleate conidia. This makes more difficult the study of a variety of problems, such as the isolation of mutants, the measurement of mutation rates, the segregation of nuclei from heterocaryons (Gossop, Yuill, and Yuil[9]; Pontecorvo[10]; Raper and Fennell[11]), the detection of cytoplasmic inheritance, and the selection of recombinants (Pontecorvo and Roper[12]). However, the results reported in this paper will give a certain amount of interesting cytological and genetic information, and encouragement for the field of industrial fermentation in this country.

The preliminary reports[13-24] [7] of our studies have been presented in the Journal of the Agricultural Chemical Society of Japan (1951–1955, Part 1–13). The important results described in those reports and further informations are summarized in the present report.

Brieriey[25] divided into three principal types:

1) Modification: non-heritable differences caused by different environmental conditions.

2) Continuous variation: heritable differences characterized by a gradual change through successive generations.

3) Discontinuous variation: heritable differences characterized by the suddenness of their appearance.

The term "variation" as used by the authors includes both "continuous variation" and "discontinuous variation" as used by Brieriey. "Natural Variation" is applied to variation occurring in the absence of any artificially imposed stimuli. Variation is regularly encountered among so called "Wild strains" isolated from soils or commercial Koji-materials and among "Culture strains" preserved as stock cultures in the laboratory. The present paper deals with natural variation in monospore lines of Koji-molds. There arise a multiplicity of forms through gradual changes in cultural appearance, morphological characteristics, and probably also in physiological properties.

Upon comparison of the characteristics of two strains at the extremes of variation within a line of single origin, it is entirely probable that one would describe them as distinct species. However, the difference between these extremes, is bridged completely by a series of intermediate or intergrading forms showing all degrees of variation.

Variants appear frequently as colony sectors or as overgrowths. When isolated in pure culture they may or may not retain their distinguishing characteristics. Occasionally variants obtained from laboratory cultures differ from the parent strain sharply, and they prove constant in subsequent culture. Such variants may be considered "spontaneous mutants".

It is important to decide what characters should be chosen as taxonomic criteria. Sakaguchi and Yamada's[26] classification of Koji-molds is based upon three morphological characteristics: texture of conidial walls (echinulate~smooth), texture of conidiophore walls, and size of conidia. This paper also considers whether variation results in such basic character differences.

There exists a considerable literature on natural variation in *Penicillium* (Baker[27]; Pontecorvo[10]; Raper[28]; Sansome[29]; Arima[30]), but in *Aspergillus* little work has been done except with a strain of an ascosporic species, *Asp. fischeri* (Greene)[31].

METHODS

(1) Strains tested:

Wild strains—Ten strains of *Asp. oryzae* were isolated from soils by Iizuka (unpublished): aX, bX, cX, eX, fX, gX, hX, J-160, E-29, and FG.

Six strains were isolated from spontaneously fermented Hattchomiso-Koji, a sort of soy bean-Koji, by Okamoto[32], and described as *Asp. flavus* Link as defined by Thom and Raper: A-3-2, A-3-8, A-24-1, A-24-3, A-5, and A-88-1.

Culture strains—About two hundred yellowish-green strains of Koji-molds were isolated from various Koji products by Sakaguchi and Yamada[33], and have been preserved by mass transfer on Koji-extract agar slants twice a year over a period of about ten years. They were divided into two species, based on the character of the conidial walls and of the conidiophore walls: *Asp. sojae* has echinulate conidia and smooth conidiophores, and *Asp. oryzae* has smooth or rough conidia and rough conidiophores. The latter species was further subdivided into three varieties according to conidial size:

i) *Asp. oryzae var. microsporus* has conidia less than 6 μ.
ii) *Asp. oryzae* s. str. has conidia 6–8 μ.
iii) *Asp. oryzae var. magnasporus* has conidia more than 8 μ.

42 of these strains were used for this study. *Asp. oryzae var. microsporus* (5 strains): F, G, SH15-3, SH22, and S-3-4.

Asp. oryzae s. str. (15 strains): H, A, B, S1-2, S1-9, S4-15, S4-18, S4-20, S6-5, T8, S7, K.B., S1-3, H1-2, and O20-3.

Asp. oryzae var. magnasporus (17 strains): A5-3, A1-5, S4-6, S3-2, S2-3, SH8-2, SH8-4, SH31, SH34, T21, SH13, SH33, H1-1, SH24-1, A5-4, A5-7, and SH15-2.

Asp. sojae (5 strains): SH10-1, SH10-3, SH10-6, SH10-7, and SH25.

The above 58 strains were divided into three colony types on the basis of conidial and mycelial production: C (conidial type)-whole colony covered with conidia and no aerial mycelium; M (mycelial type)-colony covered with abundant whitish aerial mycelium and a few conidia; X (mixed type)-colony composed of conidia and aerial mycelium in various proportions.

(2) Media:

Synthetic minimal medium: Czapek's agar with glucose as a C-source. This medium has been extensively used for cultural or morphological studies, isolation of biochemical or morphological mutants, isolation of heterocaryons, and so on.

Natural media: (i) Koji-extract agar. This solution is made by saccharification of a mixture of Koji and distilled water (1:5) at 55–60°C for 6–8 hours, followed by filtration and dilution to Ballg. 8°. (ii) Yeast-extract agar. After heating 200 g. of pressed yeast in 1000 ml. distilled water at 100°C for 30 minutes the extract is prepared by filtration of the mixture and subsequent supplementation with 50 g. glucose and 5 g. peptone.

A great majority of Koji-molds sporulate more quickly and abundantly upon natural media than upon Czapek's agar. For this reason natural media are very useful where a large quantity of conidia is desired. Furthermore, fruiting structures which form on natural media after short incubation facilitate the characterization of colony type in some strains. This is helpful for isolating rare variant types.

(3) Single spore Culture:

As a means of preliminary purification of the original parent strains, isolation from dilution plate cultures was repeated three times. The first transfer (T_1) was made by Lindner's small drop culture method. Several drops of an extremely thin conidial suspensions were placed on a cover-glass under sterile conditions. Each of these drops was then examined with the aid a microscope, and only drops having a single conidium were transferred to fresh agar slants. Therefore there can be no doubt that the resulting colony arose from a single conidium, and represents a single species or strain. After the first transfer several hundred subcultures were made by the technique of Arima[30], with which single spore isolations can be made. Conidia from strains cultured on Koji-extract agar slants for 7 days were suspended in sterile distilled water. Uniform conidial suspensions were prepared by vigorous shaking with a vibrator for 15 minutes and then by filtering with NO. 1 filter paper or NO. 2 glass filters. After counting the number of conidia with a hemocytometer, dilutions were made by adding 1 ml. of the conidial suspension to 9 ml. of sterile water. This was continued until we obtained the appropriate conidial density giving 40–70 colonies per plate. For plating, 1 ml. of diluted conidial suspension and 10 ml. of medium, at a temperature not exceeding 45°C, were poured into each petri dish. The plates were then incubated for 40–45 hours at 30°C; colonies picked at random were spot transferred onto Czapek's agar and Koji-extract agar slants at the same time. For each strain tested 400–500 colonies were isolated. After 48 hours' additional incubation of the above plates, the number of colony types segregated from each culture strain was counted by inspection. More than 95 per cent of the conidial suspensions prepared by the above method were proved by microscopic inspection to contain no conidial clumps. When single spore cultures are to be made in large number it becomes necessary to use an isolation method that is both precise and expeditious. The technique described above has proved to give most satisfactory results.

Strains in which no variants were found among about 100 colonies were

further examined by single spot-culture. A loop of conidia was removed from a stock culture purified by repeated single spore culture, and inoculated onto a plate of Czapek's agar. Satisfactory transfers have been made by using a sharp wetted nichrome wire as an inoculating needle. After 7–10 days' incubation, infrequent variants (color or mycerial type variants), were isolated from sectors or patches. These were subsequently analyzed by the described technique, using single spore cultures.

RESULTS

(1) Characterization of colony Types:

Rigid controls were used to minimize any alteration in colony character in response to changes in the composition of the medium or to other environ mental factors (age, temperature, etc.). In this study each strain was grown on Czapek's agar at 30°C for 7 days.

Colony types (Photo. 1), divided on the basis of such cultural characteristics as amount of sporulation, amount of aerial mycelium, color of conidial head, height of colony, and general habit of growth, are as follows:

(i) They were first divided into 6 types according to sporulation and floccosity: C—the heaviest sporulation; M—the most abundant floccosity; Cm—similar to C but a little more floccosity; Cmm—more abundant floccosity than Cm; MC—intermediate between C and M; and Mc—similar to M but a little more sporulation.

(ii) The above 6 types were subdivided according to the color of the colony. The citation of color is based upon Ridgway's[34] "Color Standard and Nomenclature". This terminology is also used consistently for the description of color mutants. Color variants are described by adding a number to the colony types: O is added for Parrot Green (VI), 2 for Yellowish Oil Green (V), 3 for Citrine (IV), 4 for Reed Yellow Olive (XXX), and 5 for Deep Colonial Buff-Honey Yellow (XXX).

(iii) Colony types also were divided according to growth rate "R" for restricted growth rate, is added to the previous symbols were appropriate (CR, MR, MCR, and so on).

(iv) M and MC were further subdivided according to the height of the colony. "I" describes the taller, "II" the shorter types of growth.

Additional colony types are as follows:

(i) St (Sterile type)-colony growth is flat and compact. There is little sporulation upon all media tested. This type was found very frequently among induced mutants.

(ii) Nit—very sparse growth upon Czapek's agar. This typeis unable to utilize nitrate nitrogen, but able to grow and sporulate normally upon containing ammonia nitrogen. Hence it is presumed to be a biochemical mutant requring reduced nitrate.

(iii) N—no growth on Czapek's agar, but growth on Koji-extract or yeast-extract agar.

(iv) LS—semi lethal; growth ceases immediately after germination. Ford[35] described lethal colonies of *Chaetomium globosum* obtained after UV or X-ray treatment. The colony size is less than 1 mm. in diameter when growth ceases, and the character is inherited. Beadle and Tatum[36] found that slow growing colonies, or colonies of *Neurospora* with rudimentary mycelia, germinated on complete medium, but could grow only about 1 mm. These types seems to be similar to our LS types which are frequently found among morphological mutants of Koji-molds.

(2) Variability in Each Strain:

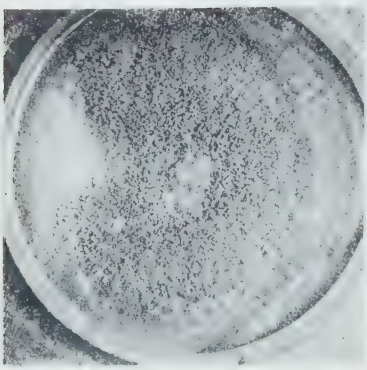
The results of differences in variability among 58 strains are summarized with regards to source and colony type (Table 1). It will be seen that

Table 1. Distribution of the Constant and Inconstant Strains among the Koji-molds Tested in Relation to Their Source and Colony Type.

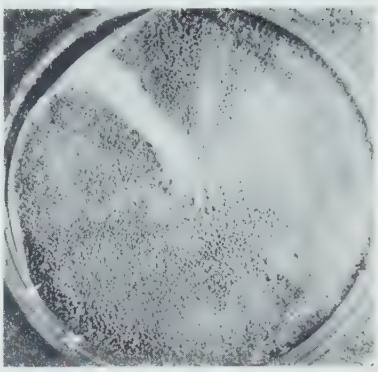
Origin		No. of Strains	No. of Constant Strains	No. of Inconstant Strains	Colony Type		
					C	M	X (Cm, MC, Mc)
Wild Strains	from Soil	10	3	7	3	0	7
	from Hattchomiso-Koji	6	4	2	4	0	2
Culture Strains	*Asp. sojae*	5	5	0	5	0	0
	Asp. oryzae var. microsporus	5	5	0	3	2	0
	Asp. oryzae s. str.	15	8	7	3	5	7
	Asp. oryzae var. magnasporus	17	4	13	0	4	13
Totals		58	29	29	18	11	29

there exist two groups in the strains of Koji-molds investigated. One group consists of inconstant strains, which are very liable to produce natural variants, and the other consists of strains which remain constant through successive single spore cultures. Most of the inconstant strains have mixed-type colonies (X) bearing various proportions of conidia and aerial mycelium (Cm, Cmm, Mc, and MC). They generally form large conidia and belong to *Asp. oryzae var. magnasporus* but sometimes they belong to *Asp. oryzae* s. str. The colonies of the constant strains show abundant conidial formation and smooth surface (C-type), or the plentiful production of mycelium (M-type). They form mostly small conidia, and belong to *Asp. oryzae var. microsporus*, but sometimes to *Asp. oryzae* s. str. The strains of *Asp. sojae* were C-type, and exceedingly stable in their colony characters. The conidia were comparatively uniform in size and similar to those of the medium strains (*Asp. oryzae* s. str.).

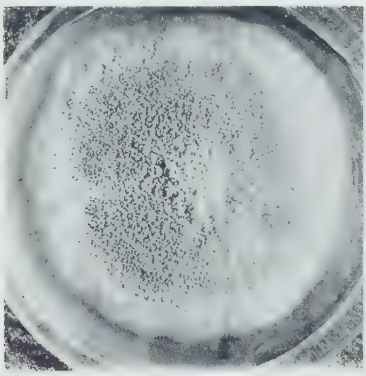
Photograph 1. Colony Types of Natural Variants



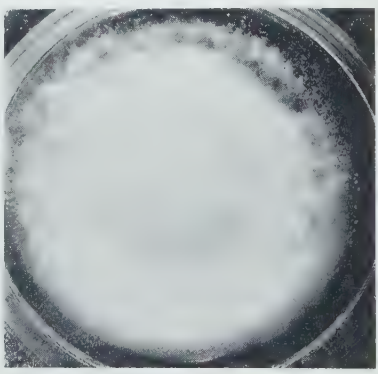
Asp. oryzae aX (Cm-type)



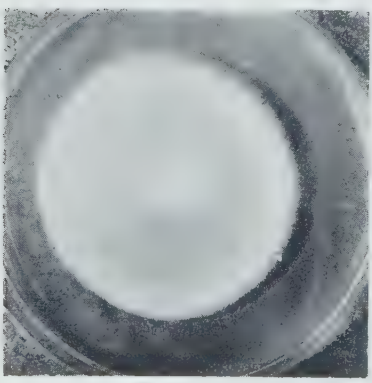
Asp. oryzae eX (Cm-type)



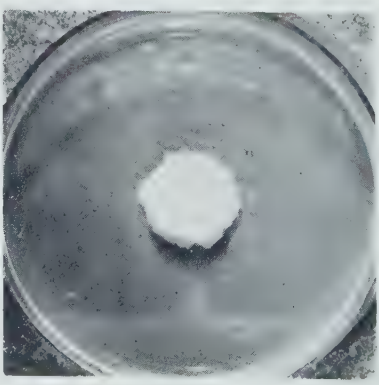
aX C-type



aX -M-type



aX--X (MC)-type



aX—MR-type

Table 2. Segregation-ratios (%) Obtained by Pedigree-Culture of Each Segregant from *Asp. oryzae* aX.

Generations	Segregants																No. of Cultures
	X (Cm)					C	M				MR			MC			
	Cm	C	M	MR	N		M	MR	MC	N	M	MR	N	MC	M	N	
T_1	23	77															65
T_2	72	26			2	100											317
T_3	80	16			4	100											639
T_4	94	0	0	1	5	100											497
T_5	93	0	0	6	1	100	87	13		0							211
T_6	56	15	26		3		68	10		22							263
T_7	36	15	49		4		71	22		7							282
T_8							62	16		22							165
T_9							38	0	41	21	92	2	6				140
T_{10}							89	7	0	4	89	7	4	88	2	10	104
T_{11}							96	0	0	4	0	96	4	98	0	2	146
T_{12}							98	0	0	2	2	96	2	96	0	4	91
Totals																	2920

Intervals of Culture: → One Week
⇢ Six Months

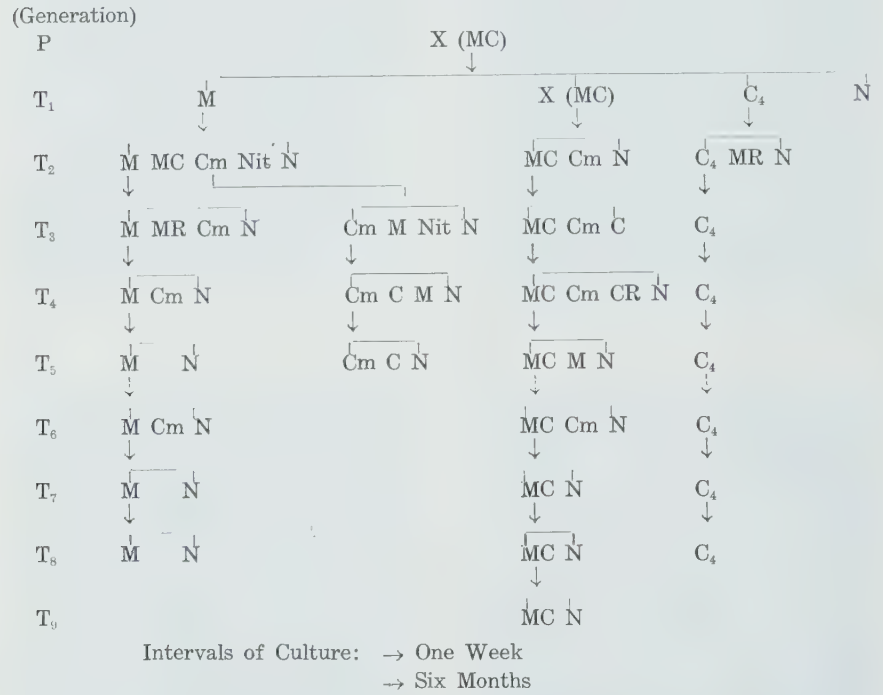
(3) Pedigree Cultures:

Pedigree cultures of 8 comparatively inoconstant strains were made in order to determine whether or not there are definite segregation ratios for each colony type, and whether or not segregants occurring in the course of the subsequent single spore cultures are stable.

These cultures were made at intervals of 10–14 days for 8–10 generations. As shown in Table 2, no fixed segregation ratios were observed. After successive single-spore cultures, all C-types were found to remain constant, and M-types were found to be stable except for those segregating into MR- and N-types. On the other hand, X-types (Cm, CM, Mc, etc.) continued to give rise to several colony types including both inconstant and constant forms. It is therefore suggested that the two former (C and M) types are homo-types, and the latter (X) types are hetero types composed of various proportions of two or more homo-types. Among all 8 inconstant strains the behavior of the progeny is essentially the same.

Fig. 1 shows the results of the pedigree culture of *Asp. oryzae var. magnasporus* A5–3.

Fig. 1. Pedigree Culture of *Asp. oryzae var. magnasporus* A5–3.



Among the segregants the inconstant strains ordinarily become constant after 5–6 generations in pedigree culture. However, upon single spore analysis of old cultures of such fixed strains, which have been kept for about six months at room temperature on Koji-extract agar slants, remarkable segregations were again found. The C-types segregating from X-types are rarely lost due to rapid overgrowth of the M-type, but they show a tendency toward reversion to the original X-types.

DISCUSSION

Greene found variant progeny of two main types among 448 single spore cultures of *Asp. fischeri*: (1) cultures producing very large, scattered perithecia, and (2) cultures producing conidial structures in profusion, but forming few perithecia. The first was variable; conversly, the second was fairly stable. Unfortunately Greene did not do any cytological work, nor was he consistent in his use of conidia or ascospores as the sources of his lines. Neither was any attempt made to recombine strains that showed departure from the original types. A similar phenomenon was described

by Hansen and Smith[37] and by Hansen[38]. They called it a "dual phenomenon" referring to a culture composed of two distinct individuals. Such dual fungi give rise to three culture types upon single spore isolation. One, producing abundant mycelium and few condia, is called the M type, another producing many conidia and relatively less mycelium, is termed the C type, and a third type is named MC, since it is composed of both M and C. The dual phenomenon was also described by Wilhelm[39] in the *Dermatophytes* and by Robbins and McVeigh[40] in *Trichophyton mentagrophytes*. Whether hetero-types of Koji-molds are composed of only two components, or more than two, will be discussed in a later paper presenting the results of successive single spore analysis of the "large conidial strains".

In this study 58 original isolates of Koji-molds from different sources were analyzed by single spore culture; it was found that 29 isolates (50 per cent) were variable. Repeated single spore cultures of these inconstant strains and further analysis revealed quite a striking range of variation. It is a very difficult matter to investigate variation under natural conditions, which are much more complex than laboratory conditions. However, it is suggested that such variation exists in Nature also, and that many of the strains isolated as homo-types had arisen in this manner. This diversity of morphology of Koji-molds may be due to mutation alone, but their instability may be combined with some other mechanism such as heterocaryosis. Detailed studies concerning this problem will be described in the near future.

SUMMARY

(1) Natural variation in monospore lines of Koji molds (*Asp. oryzae* and *Asp. sojae*), isolated from commercial Koji material or soil and from laboratory stock cultures, has been observed.

(2) We can divided the 58 strains of Koji molds investigated into two groups; one group consists of inconstant strains which are very liable to produce natural variants, and the other consists of strains which remain constant through successive single spore culture.

(3) The inconstant strains develop colonies bearing various proportions of conidia and aerial mycelium (X-type). They generally form large conidia (*Asp. oryzae var. magnasporus*) but sometimes medium sized conidia (*Asp. oryzae* s. str.), which produce large conidia occasionally.

The colonies of the constant strains show abundant conidial formation and smooth surfaces (C-type). The conidia are mostly small (*Asp. oryzae var. microsporus*) but sometimes medium in size (*Asp. oryzae* s. str.).

(4) The colony types of the variants are as follows:

C (Conidial type, whole colony covered with conidia), M (mycelial type), R (restricted in growth rate), St (sterile type, little sporulation on all media tested), Nit (requiring reduced nitrate, very faint growth on

Czapek's agar), and LS (semi lethal, growth cease immediately after germination).

(5) Pedigree cultures of the 8 inconstant strains have been made, but no definite segregation ratios for each variant type have been recognized through successive generations.

(6) The LS and N types commonly occur spontaneously from the M-type.

ACKNOWLEDGEMENTS

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HEREDITARY VARIATION AND GENETIC RECOMBINATION IN KOJI-MOLDS (*ASPERGILLUS ORYZAE AND ASP. SOJAE*)

II. MORPHOLOGICAL MUTATION

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Iguchi investigated in detail a large number of morphological and biochemical mutants[1-2] obtained after treatment of *Asp. sojae* with ultraviolet light, X-rays, and nitrogen-mustard.

Preliminary work[3] presented in 1951 by one of the authors, was carried out with two objects. One was to determine whether the constant C- and M-types which were obtained from one of the "Wild strains" of *Asp. oryzae* aX by successive single spore cultures, revert to the parent (X-type) after ultraviolet irradiation. The other was to study the relation between the occurrence of natural variants and induced mutants and to learn the extent and direction of variation.

METHODS

(1) Origins:

C- and M-types[4] obtained from *Asp. oryzae* aX. Both strains were subjected to single spore culture for five transfers, but no other colony type segregated. It is therefore clearly confirmed that these strains are homotypes.

The strains used for obtaining color mutants are as follows:

Asp. oryzae K.B. (wild type), K-25 (methionineless mutant which was produced by ultraviolet treatment of K.B.) *Asp. sojae* K.S. (wild type), X 793 (leucineless), and X-398 (histidineless).

(2) Irradiation:

Conidia taken after 7-10 days' incubation on Koji-extract agar slants, were suspended in 20 ml. of sterile distilled water, filtered through a NO. 2 glass filter, and diluted as necessary to yield concentrations in the neighbourhood of $3\text{-}5\times10^6$ spores/ml.

The treatment for obtaining morphological mutants is as follows: an ultraviolet lamp delivering more than 95 per cent of ultraviolet energy at a wave length of 2537 A° was used; 10 ml. of conidial suspension was

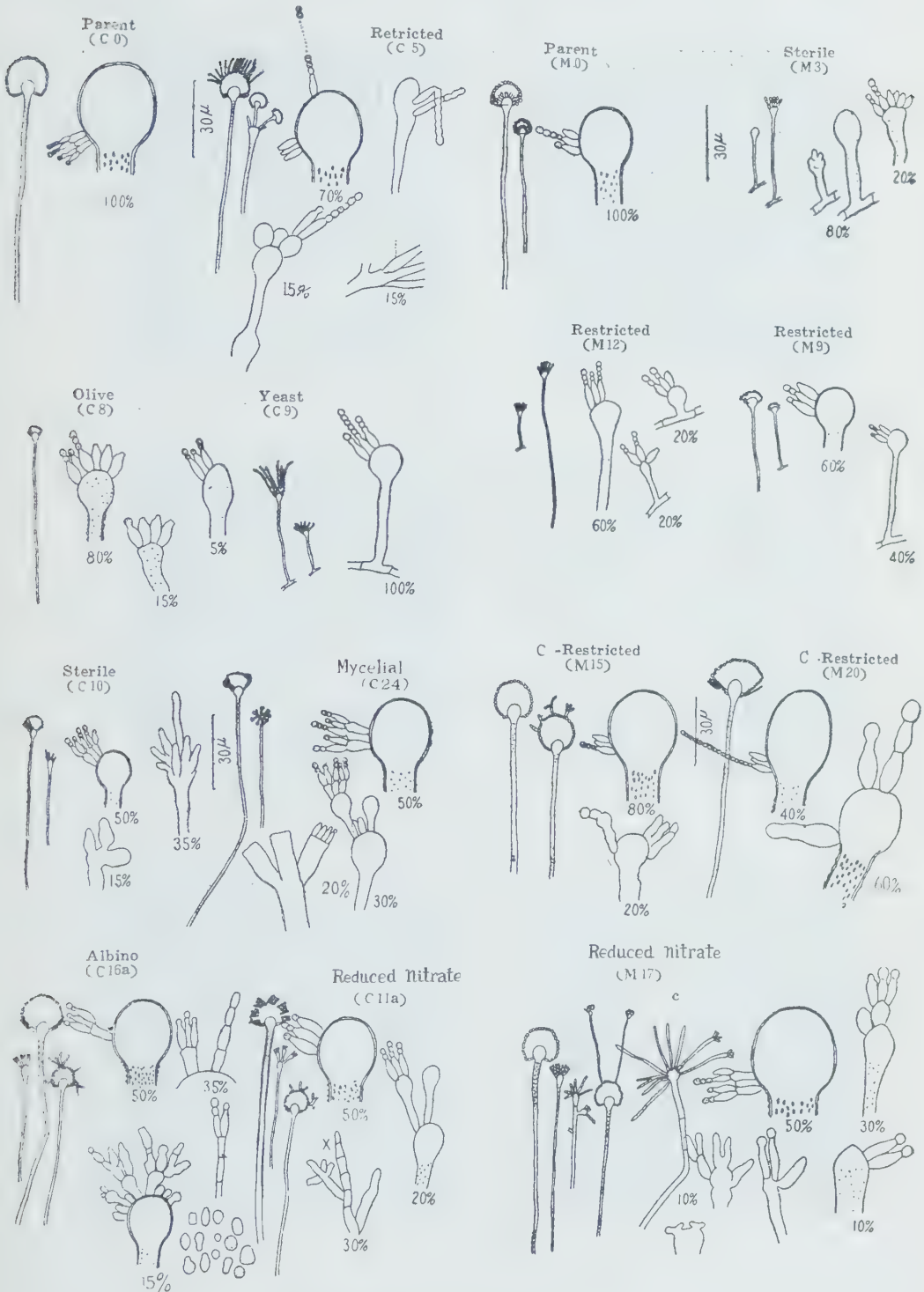


Fig. 1. Conidial Structures of Induced Mutants from C- and M-types of *Asp. oryzae* aX.

transferred to an open petri dish (6 cm. diameter), and was irradiated to an open petri dish (6 cm. diameter), and was irradiated under varying conditions (at distances of 25–100 cm. and times of 0.5–60 min.).

The suspension was gently stirred during irradiation. One ml. samples of the suspension being irradiated were removed periodically. They were plated out in quadruplicate at a concentration which would give separate colonies on Czapek's or Koji-extract agar. Colonies appeared after 40–48 hours' incubation, were isolated by rough visual selection in the manner previously described. Survival determinations were made by counting the colonies appearing on Koji-extract agar.

RESULTS AND DISCUSSION

(1) Characterization of Colony Types:

Isolations of 110 strains out of 731 surviving conidia of the C-type and of 186 strains out of 810 surviving conidia of the M-type were made onto Czapek's agar slants. Among the 296 isolates, 85 strains were nearly normal in culture appearance and the rest were obvious morphological mutants. Representative strains of both groups (20 strains of the almost-normal type and 95 strains of the mutant types) were grown individually Koji-extract agar and Czapek' agar as giant colonies.

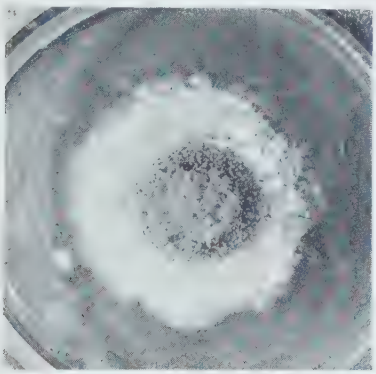
The majority of mutants could be classified into fairly definite groups by the use of the following characteristics: color of colony, production of conidia and aerial mycelium, rate of growth, secretion of pigment, etc.

The mutant types obtained from the C- and M-type strains are summarized in Table 1. This table also gives colony types which arose spontaneously from both the original strains. The characters of the two original parent strains are as follows.

C-type strains: Colonies grow rapidly on Czapek's agar. After 6 days' incubation at 30°C they are 7–8 cm. in diameter. They show heavily sporing surfaces, without aerial mycelium. The massed conidial heads give a distinctive color and colse texture. Colonies are uniform in color (Parrot Green VI) or with central area somewhat lighter (Yellowish Oil-Green V). Conidial heads are large and radiating, with separate, rather than adhering, chains of conidia. Submerged mycelium is colorless, hyaline, and thin-walled. Reverse of colonies is at first yellowish, changing to orange with age due to secretion of Kojic acid. Conidiophores mostly arise from submerged hyphae, and have rough walls, almost spiny in appearance. Vesicles are sub-globose, 20–50 μ in diameter. Conidia are almost globose, 2.5–5 μ in diameter, with thick roughly pitted walls. The morphological description of the C-type strain is given in Table 3, together with its derived mutants. The Table also describes the original M-type strain and the mutants derived from it.

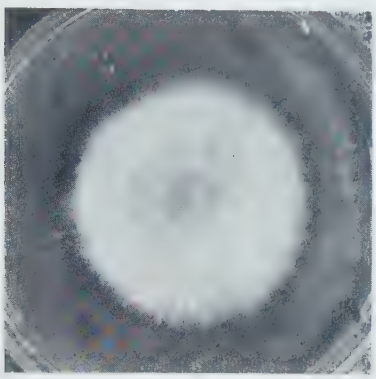
M-type strains: Colonies of the M-type have the same growth rate as those of the C-type. The strains are characterized by white floccose colonies

Photograph 1. Colony Types of Induced Mutants



C6 Restricted type

C_1 Restricted type



C10—Sterile type

C9- Yeast type

C16a—Albino type

M17—Reduced nitrate

Table 1. Comparison of Colony Types between Natural Variants and Induced Mutants.

Colony Type		Natural Variants	Induced Mutants	
			from C	from M
Conidial	C	+	+	+
Mycelial	M	+	+	+
Mixed	X (MC, Cm)	+	+	+
Sterile	St	+	+	+
Yeast		−	+	+
Restricted	CR. MR.	+	+	+
Heavy		−	+	−
Albino	Al	+	+	+
Yellow	Y	+	+	+
Brown	B	+	+	+
Olive	Ol	+	+	−
Maroon-exudate		−	+	−

+: Occurrence. −: no Occurrence.

Table 2. Relationship between the Percentages of Different Types of Natural Variants and Induced Mutants from the M-Type of *Asp. oryzae* aX.

Colony Types	Natural variants	Induced Mutants					
		Various Conditions of UV Treatment					
		100cm.			50 cm.		25 cm.
		5 Min.	10 Min.	20 Min.	10 Min.	20 Min.	10 Min.
M	92	81	83	42	79	30	47
MR	1	10	2.4	26	3.4	37	25
St	1	2	2.4	6	0.6	3.5	6
N	6	7	9.0	26	7.0	7.0	2
Nit	0	0	0.4	0	0	0	0
C	0	0	0	0	0.9	1.0	2
CR	0	0	1.6	0	7.0	10.0	9
Y	0	0	0.2	0	0	3.5	0
B	0	0	0	0	1.1	6.0	6
X (MC)	0	0	1.0	0	1.0	0	0
Total variants	7	19	17	58	21	70	53

in which conidial heads are frequently borne upon aerial hyphae; These heads are generally lighter than those of the C-type (Deep Colonial Buff-Honey Yellow XXX). They are normal in their structure, and usually somewhat compact, and columnar. Most of them are small, but a few of

them are fairly large. Conidiophores with pitted or rough walls are usually short, varying from 300 μ to 1000 μ. Vesicles are mostly under 20 μ in diameter. Conidia are almost globose, 3.3–6.7 μ in diameter with thinner walls than those of the C-type.

The cultural and morphological characteristics of the mutant types are as follows:

1. Almost-normal type: Here we include 35 strains out of 110 suspected mutants from C-type, and 50 strains out of 186 from the M-type. This type is similar to the parent in colony appearance; however, some strains obtained from the C-type show a delay in sporulation on Koji-extract agar, and have malformed secondary sterigmata.

2. Restricted type: A restricted colony reaches a diameter of 1.5–5.5 cm. after 6 days' incubation. Some strains derived from the C-type have frequently malformed large sterigmata or heads witn long conidial chains. The strains derived from the M-type usually show small heads or vesicles and especially short conidiophores.

3. Albino type: This differs from the original type in the absence of pigment in the conidia. Colonies either remain white or, after about 14 days' cultivation, change to a color near Naples Yellow (XVI) or Sulphur Yellow (V). In morphological characteristics one is typical and the other is exceedingly atypical. For example, in the latter the primary and secondary sterigmata are usually atypical in size and shape. Individual conidia are not completely differentiated: they are essentially the same in morphology as normal sterigmata, and they form long chains (Fig. 1). A type similar to this was found by Yuill and Yuill[5] in a culture of *Asp. niger*, and called "*Cladosarum olivaceum*"; the secondary sterigmata thrust out cells resembling themselves, instead of producing conidia. The strains of such atypical albino types produce conidia in a great variety of shapes including ovate, elliptical, cylindrical, pyriform, and fusiform.

4. Brown type: The color of the colonies is Sudan Brown (III). The strains arising from the C-type are almost the same as the parent in colony appearance and morphological characteristics. On the other hand, the strains from the M-type produce conidia with strongly echinulated walls and a rather large diameter (4.2–11 μ).

5. Yellow type: This differs from the original type only in the color of the colony (Yellow Ocher XV), which at maturity is Citrine (IV).

6. Olive type: Colonies are Deep Olive Buff (XL). The conidial heads are comparatively uniform and small in size. About 20 per cent of the vesicles examined are clavate or similar to those of *Citromyces* in shape.

7. Sterile type: This has almost the same colony appearance as some natural variants. Colonies grow rapidly on both Czapek's and Koji-extract agar. Conidial heads are not only limited in number but are commonly atypical in pattern; conidiophores may fail to develop vesicles, or vesicles may fail to produce sterigmata and conidia.

8. Yeast type: This is nearly the same as the "Leathery" type

described by Hollaender *et al.*[6] in *Asp. terreus*. The colonies are very like yeast; close-textured, strongly wrinkled and conspicuously restricted in growth rate. The colonies are almost entirely composed of a mass of mycelium, which bears only vesicles or a few, small atypical conidial structures.

9. Mycelial type: This type, obtained from the C-type, resembles the original M-type in colony characteristics. However, conidial heads and vesicles are frequently atypical, and conidia occur in a great variety of sizes (2.5–8 μ).

10. Heavy type: Sporulation is much more abundant than in the original C-type. The colonies are somewhat high and compact. The margin is smooth, and the reverse is leathery. Morphological characteristics are generally normal. This type is the same as one obtained by Iguchi[7] from *Asp. sojae*.

11. C-type: This arose from the M-type but is similar to the original C-type in its appearance; in morphological characteristics, however, there are differences between them. Vesicles have thin-walls and vary considerably in diameter (9–42 μ). Conidiophores also are thin-walled and rather slender.

12. CR-type: This also was produced from the M-type and it differs from the mutant C-type in growth rate. In addition, vesicles are rather large and globose or elliptical in shape. Extraordinarily enlarged sterigmata were often encountered, as shown in Fig. 1.

13. Maroon-exudate type: This mutant was obtained from the C-type. The colony is considerably restricted and Reed Yellow~Olive-Yellow (XXX) in color. The strain secrets an extensive amount of maroon pigment into the medium. Conidial heads are generally small and somewhat atypical. There is little difference in growth on Czapek's and Koji-extract agar. The presence of excessive amounts of pigment may indicate some physiological change in this form.

14. Reduced nitrate type: On Czapek's agar colonies, including 5 strains from the C-type (Clla, b, c, d, and e) and 3 strains from the M-type (M11, M13, and M17), grow as rapidly as the original types, but very thinly. The colonies consist of sparse, submerged mycelium which gives rise to scattered conidial heads. Upon Koji-extract or Malt-extract agar all colonies appear normal. The mutant character is an inability to utilize nitrate nitrogen. In this type, there are some typical and some atypical strains, the latter frequently showing malformed conidial heads and sterigmata.

Both parent strains (C- and M-types) produced the same kind of mutants, except for the Maroon exudate, Heavy, and olive mutants which are derived from the C-type only. Furthermore, among natural variants and induced mutants the same colony types were found except for Maroon exudate and Heavy. The only mutant we found which was not described by Iguchi[7] is Maroon-exudate.

There was no spontaneous occurrence of color mutants (albino, yellow,

and brown types), although from 1250 to 8000 colonies in each of five strains were examined by single spore culture. However, these mutants were induced with rather high frequency by ultraviolet irradiation. In the previous section we described color variants from *Asp. oryzae* cX (brown-1/1214), from *Asp. oryzae var. magnasporus* A5–3 (yellow-1/672), and from S2–3 (albino 1/1700) by succesive single spore cultures. Color variants were also isolated from sectors of *Asp. oryzae* bX and *Asp. oryzae* fX. All of these color variants were proved to be stable through repeated transfers in single spore culture. It is accordingly recognized that they are spontaneous mutants. The strains in which spontaneous color variants occurred were fairly stable, while the other five strains described above were much more so; the former strains showed the segregation of many colony types in addition to color variants, but the latter strains did not produce either color variants or any other segregants. As a matter of fact, the tendency to mutate spontaneously seems to be itself genetically controlled, as has been shown by Stakman[8] in *Ustilago zeae*.

(2) The Frequency of Mutation:

The proportion of morphological mutants among survivors, was 15 per cent (110/731) from the C-type and 23 per cent (186/810) from the M-type strain under a single irradiation condition. The former mutation frequency is like that reported by Iguchi[7] for *Asp. sojae* K. S. (14.5 per cent). According to the amount of irradiation given, 90–99 per cent of the conidia of the M-type strain were killed, and among the survivors, from 17 to 70 per dent showed cultural changes (Table 2).

The frequency of spontaneous mutation is undoubtedly very low, but it has not been satisfactorily measured in our strains. From the above results, however, it is clear that the gross frequency of mutation can be considerably increased by exposure to ultraviolet light.

(3) Stability of Mutants:

Although the changes studied are suspected to be chromosomal mutation, it is impossible to decide whether this is the case, because Koji-molds are imperfect fungi and crossing with the parent is not possible unless a parasexual mechanism can be demonstrated. By repeated transfer on standard Czapek's agar we attempted to determine whether these altered characteristics are inherited, and therefore stable.

The morphological characteristics of Almost-normal, Restricted, Sterile, Heavy, Yeast, and Maroon-exudate type mutants have remained stable through eight transfers, with a few exceptions: Sterile, Yeast, and some Restricted types have given rise to reversions to the parental types. The Reduced nitrate, Mycelial, Olive, and X (MC) mutants are very liable to give rise to parental or other types at the first transfer. It was previously recognized that from the constant strains with homocaryotic conidia these heterocaryotic mutant strains[9] could be produced by UV irradiation.

This phenomenon will be described in detail in the near future.

The color mutants of *Asp. sojae* proved to be remarkably stable after further ultraviolet irradiation; i.e., there was no reversion to wild type either spontaneously or artificially, though albino-type mutants were produced from yellow and brown-type mutants. Hence color mutants are among the best materials for genetic analysis because they are stable and they make good visible markers.

(4) The Tendency and the Extent of Variation:

The authors have been interested in determining the tendencies and the extent of variation in Koji-molds. An effort to resolve such problems has been made in the course of the present study of natural variation and induced mutation.

The most common morphological changes occurring spontaneously or artificially include color of the conidia, sporulation, and growth rate of the colony.

The color of conidial heads is one of the important characters for the classification of the members of the genus *Aspergillus*. Color variants, including albino, yellow, and brown, have been isolated from sectors or single spore cultures of *Asp. oryzae* at a very low frequency. They were quite similar to the induced mutants obtained by Iguchi[7,10-11] and the present authors except for a few morphological changes in their organs. Mutants of Koji-molds showed a rather wide continuous variation of colors through green, yellow, and brown. (It may be noted here that the green Koji-molds never produce black mutants). During the study of natural variation, the authors often found difficulty in describing the color of a colony; because of the graduation it was hard to assign colors to one of the 5 groups mentioned previously. Recently a similar situation in the genus *Aspergillus*, that is, the existence of a definite color ranges, was described by one of the authors[12].

The morphological changes accompanying restriction in growth rate and decrease in sporulation are very conspicuous. The sporulation and growth rate of color mutants, however, and normal. Extremely restricted colony type has never shown reversion in 12 successive transfers during 6 years' preservation. A few mutants such as Heavy, increased in sporulation, while the original conidial type (C) was frequently transformed into the Sterile type because of a decrease in sporulation. The Yeast type, obtained by induced mutation, is the most degenerate form in colony character. One of the authors[13] divided previously Koji-molds into two forms, anaerobic and aerobic, and he suggested that the first was degenerated form.

Sabouraud[14] referred to a definite morphological change occurring in old cultures as "Pleomorphism". This change involves the production of a white downy mycelium in the *Dermatophytes*. He considered pleomorphic growth to be a "fixed mutation". Gregory[15] considers this pleomorphism as example of the irreversible mutations or saltations so common in many

fungi rather than a process of gradual degeneration. Wilhelm[16] states that the M-type constituent arose as a mutation in old cultures of the C-type even though the culture was of single conidial origin and appeared to be genetically stable. The present authors obtained the M-type from a sector of the C type or from the C-type irradiated with ultraviolet.

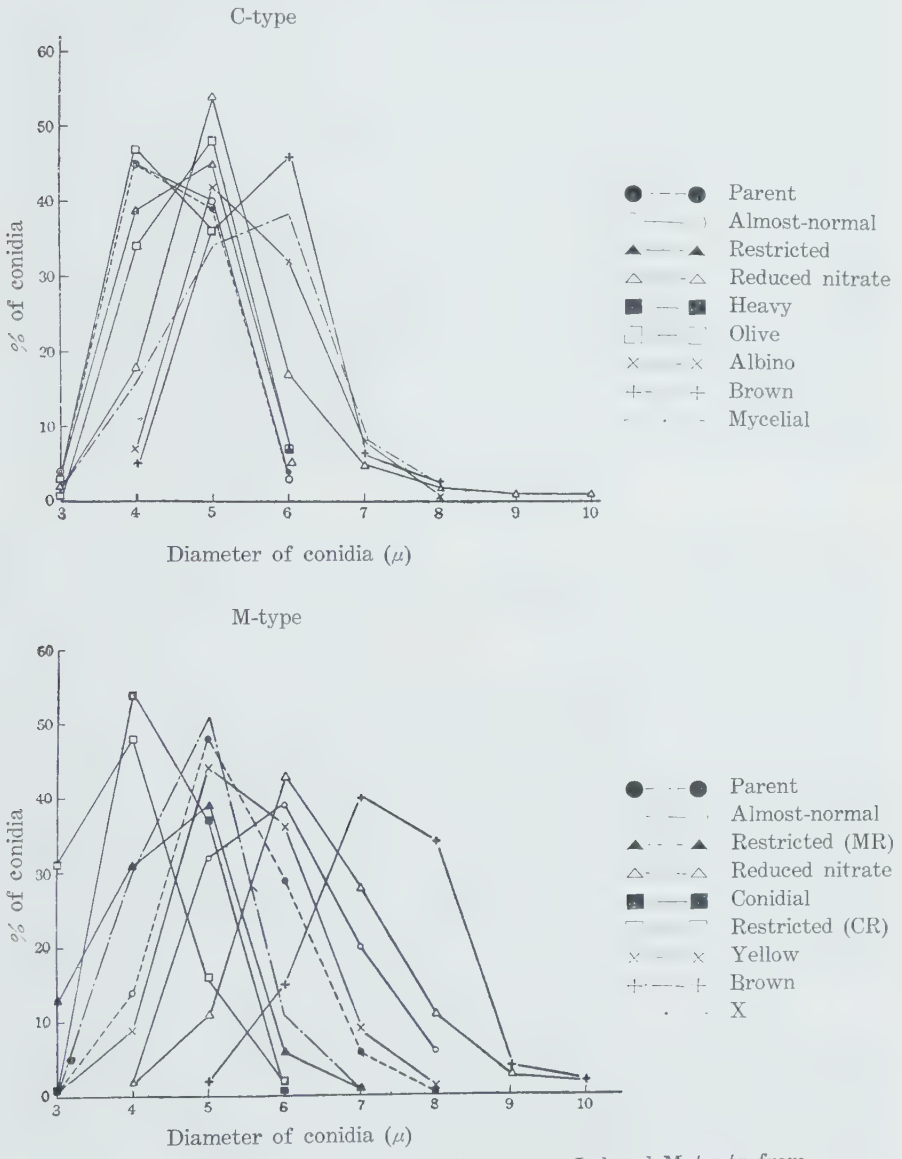


Fig. 2. Distribution of Conidial Size among Induced Mutants from C- and M-types of *Asp. oryzae* aX.

Table 3. Microscopical Observation of Representative Strains of Induced Mutants from *Asp. oryzae* aX.
(Cultured on Czapek's agar; Range of Dimensions in μ)

Type	Strain	Conidiophore	Conidial Head	Vesicle	Sterigmata	Conidia
from C-type						
Parent	C0	700~1500×8~18	80~180	20~50	5.8~ 8.3×2.5~ 4.2	2.5~ 5.0
Almost-normal	C1	700~1300×6~14	80~180	15~37	6.7~ 8.3×2.5~ 4.2	2.5~ 5.0
Restricted	C5	600~1300×7~12	60~130	13~33	6.7~ 8.3×2.5~ 4.2	2.5~ 5.8
Sterile	C10	500×1300×4~10	20~ 35	8~20	6.7~ 8.3×2.5~ 3.3	2.5~ 4.2
Reduced nitrate	C11a	700×2000×8~15	40~140	13~39	9.2~13 ×2.6~ 6.5	2.5~ 5.8
Heavy	C4	500~1000×7~10	130~180	18~36	8.3~10 ×2.5~ 3.3	2.5~ 5.8
Olive	C8	700~ 800×5~ 6	35~ 45	8~15	7.5~13 ×4.2~ 6.7	2.5~ 5.0
Brown	C19	100~2000×5~20	80~250	13~65	7.8~15.6~3.9~ 7.8	3.3~ 6.7
Albino	C16a	700~1600×5~13	100~200	14~45	7.8~10.4×2.0~ 3.3	2.5~ 5.8
Mycelial	C24	800~3500×7~22	40~140	14~45	7.8~19.5×6.5~11.7	2.5~ 8.0
Yeast	C9	35~200 ×3~ 5	60~130	7~12	6.7~ 9.2×2.0~ 5.0	2.5~ 4.2
Maroon-exudate	C7b	250~500 ×4~ 8	40~ 80	8~33	7.5~10 ×3.3~ 4.2	2.5~ 5.0
from M-type						
Parent	M0	300~1000×8~12	40~130	15~27	6.2~ 8.3×3 ~ 4.2	3.3~ 6.7
Almost-normal	M1	500~1300×5~13	40~100	15~30	6.2~ 8.3×2.5~ 4.2	3.3~ 8.3
Restricted (MR)	M9	90~ 350×3~11	20~ 50	5~21	5.8~12.5×2.5~ 4.2	2.5~ 5.8
Restricted (MR)	M12	25~ 800×3~ 7	20~ 50	4~13	5.8~11 ×3.3	2.5~ 5.8
Sterile	M3	50~ 350×6~13	20~ 60	17~18	8.2~13 ×3.3~ 5.0	3.3~ 5.8
Reduced nitrate	M17	700~2300×6~20	40~250	12~50	6.7~13.3×3.3~ 5.0	3.3~ 9.2
Conidial	M21	850~1700×4~ 8	40~100	9~42	7.5~10 ×2.5~ 4.2	2.5~ 5.0
Yellow	M18	450~1700×5~12	40~100	13~42	7.5~ 8.3×3.3~ 4.2	2.5~ 8.3
Brown	M19	1300~1700×8~17	35~ 85	8~35	8.3~10 ×3.3~ 4.2	4.2~11.0
Restricted (CR)	M15	850~1700×8~17	40~200	17~50	6.7~12.5×2.5~ 4.2	3.3~ 6.7
X	M16	850~1700×9~18	40~180	15~50	6.7~10 ×2.5~ 5.0	3.3~ 6.7

However, it is difficult to determine whether or not the M-type is a chromosomal mutation because of the absence of sexual reproduction in this strain. A later publication in these series will demonstrate the existence of a parasexal mechanism in the Koji-molds. This may allow a testing of this hypothesis.

Further information on the nature the change from C to M was obtained by Hansen and Snyder[17] by progeny analysis of crosses between haploid strains of the hermaphroditic, heterothallic fungus *Hypomyces solani* F. *curcurbitae*. They concluded that the factors for M and C are allelomorphic and inherited independently of the allelomorphic compatibility factors for A and a. Scheng and Ryan[18] showed in *Neurospora* that the line of nuclei carrying the L_2 (leucine-independent) gene developed an independent mutation to an aconidiate condition, and the gene responsible for this condition, c_1, is not linked with l_2 (leucine-dependent) gene but is on the mating type chromosome.

It has been recognized by many workers that the M-type is generally stable and irreversible. However, the M to C change was found by the authors after treatment of the M-type with ultraviolet.

Table 4. Microscopical Observation of Natural Variants from *Asp. oryzae* aX. (Cultured on Czapek's agar; Range of Dimensions in μ)

Number	Type	Strain	Conidiophore	Vesicle	Sterigmata	Conidia
1	Parent	aX	700~1700× 8 ~20	18~50	6.9~ 8.6×2.8~3.4	2.5~6.7
2	C	$AT_1$2	500~1200× 8 ~20	23~40	6.3~ 9.5×3.0~4.3	2.5~5
3	C	$BT_1$6	700~1800× 9 ~20	25~45	6.3~ 8.6×3.0~4.3	2.5~5
4	C	$BT_2$18	950~ 200×10 ~18	28~40	6.3~ 8.6×3.4~4.3	2.5~5
5	C	$BT_2$26	1300~2000×12 ~20	25~45	7.7~ 8.6×3.9~4.3	2.5~5
6	C	$AT_2$38	1200~1800× 6 ~14	15~30	6.0~ 8.6×2.6~4.3	2.5~5
7	M	$AT_5$17	40~1000× 5 ~12	11~25	7.8~ 9.8×3.3~3.9	3.3~4·8
8	M	$AT_5$50	150~ 700× 5 ~15	12~30	8.4~ 9 ×3.3~3.9	3.3~5.8
9	M	$AT_7$60	100~1000× 6 ~ 8	20~25	8.5 ×2.6~3.3	3.3~5.8
10	M	$AT_7$6	80~1250× 4 ~ 9	10~25	7.8~ 8.5×2.6~3.3	3.3~5.8
11	M	$AT_7$8	70~ 900× 4 ~11	12~25	7.8~ 9.8×2.6~3.3	3.3~5.8
12	X	$BT_1$17	160~1200× 9 ~15	20~40	6.5~10.5×2.6~5.2	3.3~6.7
13	X	$BT_1$4	100~1500× 6 ~13	17~37	6.5~10.4×4.5	3.3~6.7
14	X	$BT_3$13	100~ 800× 7 ~20	14~43	6.5~10.4×3.3~5.2	3.3~6.7
15	X	$AT_3$8	100~1000× 6 ~14	12~37	8.5~10.4×4.6~5.2	3.3~6.7
16	X	$AT_4$4	130~ 800× 7 ~12	14~28	6.0~ 7.8×2.6~3.3	2.5~6.7
17	X	$BT_1$24	300~1000× 7 ~12	5~27	7.5 ×2.2~2.8	2.5~6.7
18	X	$BT_1$18	90~1800× 5 ~13	14~33	6.0~ 7.8×2.6~3.3	2.5~6.7
19	X	$BT_1$25	150~ 800× 4 ~ 9	12~23	8.5~10.4×4 ~4.5	2.5~6.7
20	X	$AT_1$3	80~ 900× 3 ~10	7~23	7.8~ 9.2×4	3.3~6.7
21	X	$BT_3$5	170~ 700× 5 ~ 8	14~20	8.5~10.4×4.5	2.5~6.7
22	X	$BT_3$16	400~1200× 7 ~20	15~40	9 ~10.4×3.3~3.7	3.3~6.7
23	X	$BT_3$35	180~1000× 9 ~11	15~26	8.6~ 9.2×3 ~3.3	2.5~6.7
24	X	$AT_3$8	100~1000× 6 ~14	12~37	8.5~13 ×3.9~4.6	2.5~6.7
25	X	$AT_5$8	110~ 700× 6 ~13	20~40	7.2 ×3.9	2.5~6.7
26	X	$AT_5$42	180~ 650× 5.7~10	23~23	5.2~ 9.5×2.2~3.3	3.3~6.7

Table 5. Microscopical Observation of Natural Variants from *Asp. oryzae* bX. (Cultured on Czapek's agar; Range of Dimensions in μ)

Number	Type	Strain	Conidiophore	Vesicle	Sterigmata	Conidia
1	Parent	bX	700～ 850× 8～13	17～30	7.5～ 9.2×3.3	2.5～5.8
2	C	F59	500～ 850×10～16	33～42	6.7～ 7.5×3.3～4.2	2.5～5
3	C	D6	800～1000× 9～17	33～50	7.5×3.3	2.5～5
4	X	A10	850～1200× 6～15	9～32	8.3～10 ×3.3	2.5～5.8
5	X	B10	1100～1800× 8～13	13～36	8.3～10 ×3.3	2.5～5.8
6	M	H55	500～1000× 9～13	23～48	7.5～10 ×3.3～4.2	2.5～5.8
7	M	$HT_1$7	300～ 700× 6～14	14～50	8.3～10 ×5 ～5.8	2.5～5.8
8	X	$HT_1$9	500～ 800× 6～14	13～32	7.5～ 8.3×3.3～4.2	2.5～6.7
9	X	$HT_3$1	800～1300× 7～17	21～46	7.5～ 9.2×3.3	3.3～6.7
10	M	$HT_4$2	600～ 850× 9～16	25～35	8.3～ 9.2×4.2	3.3～5.8

The conidia of the C- and M-types (the constant natural variants) are of comparatively uniform size. The conidia of mutants from these strains are mostly similar in size to those of the parents, except for those of the Reduced nitrate, X (MC or Cm), Brown, and some M-types which are generally of a variety of sizes (see Fig. 2 and Table 3).

The shape and size of each organ (vesicle, conidiophore, sterigma, and conidial head) are remarkably changed by UV treatment. These changes were rarely observed in color mutants but frequently in the mutants of regressive types having restricted growth or colonies deficient in sporulation.

Observations of morphological characteristics were also made on C-types (7 strains), M-types (8 strains), and X-types (19 strains) from natural variants found in *Asp. oryzae* aX and bX (Table 4–5). In general the only difference found was in the size of the conidia. These natural variants, therefore, seem to differ to a slight degree than in basic character changes.

Asp. sojae has been distingushed from *Asp. oryzae* by the structure of the walls of conidiophores and conidia. Iguchi[19] through accurate observation of 202 mutants also finds that these two characteristics are comparatively unchangeable. As these two characters are more stable than the others, they are useful as taxonomic criteria.

SUMMARY

(1) The constant C- and M-types which were obtained from one of the "Wild Strains" (*Asp. oryzae* aX) have been subjected to ultraviolet treatment.

(2) M-type mutants have been obtained from the parent C-type, and C- and X-type mutants from the parent M-type. These mutants have proved to be the same as the natural variants mentioned above (1).

(3) The mutants that have been obtained from the C-type are as follows: 1. Almost-normal 2. Restricted 3. Sterile 4. Reduced nitrate 5. Heavy 6. Olive 7. Brown 8. Albino 9. Mycelial 10. Yeast 11. Maroon-exudate.

The mutants that have been obtained from the M-type are as follows: 1. Almost-normal 2. Restricted 3. Sterile 4. Reduced nitrate 5. C 6. CR 7. Brown 8. Yellow 9. X

(4) The conidia of the C- and M-types which are natural variants are of comparatively uniform size. Among induced mutants from the C- and M-types, the conidia of Reduced nitrate, Brown, Mycelial, and X are generally found in a great variety of sizes.

(5) Thus the morphology of Koji-molds has been remarkably changed by ultraviolet irradiation except for the structure of conidial and conidiophore walls.

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HEREDITARY VARIATION AND GENETIC RECOMBINATION IN KOJI-MOLDS (*ASPERGILLUS ORYZAE* AND *ASP. SOJAE*)

III. BIOCHEMICAL MUTATION

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Recently single biochemical mutants or color mutants of *Asp. sojae* K. S. and *Asp. oryzae* K.B. were irradiated with UV light in order to obtain multiple mutants having both a color and a nutritional marker. These mutants were used for making balanced heterocaryons[1] and for production of "heterozygous diploid" strains[2]. In the course of the study, the frequency of color mutants and types of morphological mutants were also examined.

METHODS

(1) Origins—Four strains of *Asp. sojae* and one strains of *Asp. oryzae* were used:

Asp. oryzae K.B.; *Asp. sojae*—the yellow mutant (Y_1), the single auxotrophs, leucineless x-793 (14C *leu*), histidineless x-398 (10C *his*), and histidineless albino H158 (*al his*). The first two single auxotrophs among those received originally from Dr. Iguchi proved to be comparatively stable. The albino mutant was obtained by the authors after ultraviolet treatment of strain 10C *his*.

(2) Media—Minimal medium (M.M.):

This is the simplest chemically defined medium, sufficient for growth only for the wild type strains. Czapek's agar was usually used in these experiments. Complete medium (C.M.): This usually contains a large variety of known and unknown organic compounds. In this study Koji-extract agar, from natural sources, was used as C.M.

(3) Irradiation:

Conidial suspensions were prepared in the same way as for production of morphological mutants. The procedure for obtaining biochemical mutants is as follows: A 10-Watt germicidal lamp, (Sanyo, GL-form), was used, which emits 95 per cent of its effective radiation at a wave length 2537Å. The electric current was constantly regulated with an ammeter to a strength

of 0.22A. The light source was supported 37 cm. above 15 ml. of conidial suspension, which was contained in a petri dish (10 cm. diameter).

Irradiation of each strain was carried out ten times. The 1-ml. aliquots were removed for plating purposes at prescribed intervals during irradiation between 5 seconds and 10 minutes. Survival was determined by colony counts; an average was obtained from 4 plates in each case. The average survival ratios for each class sampled were plotted on semi-log paper. Except for the albino strain, all mutants showed survival curves that were nearly the same as that of the wild-type, as shown in Fig. 1. Survivors of

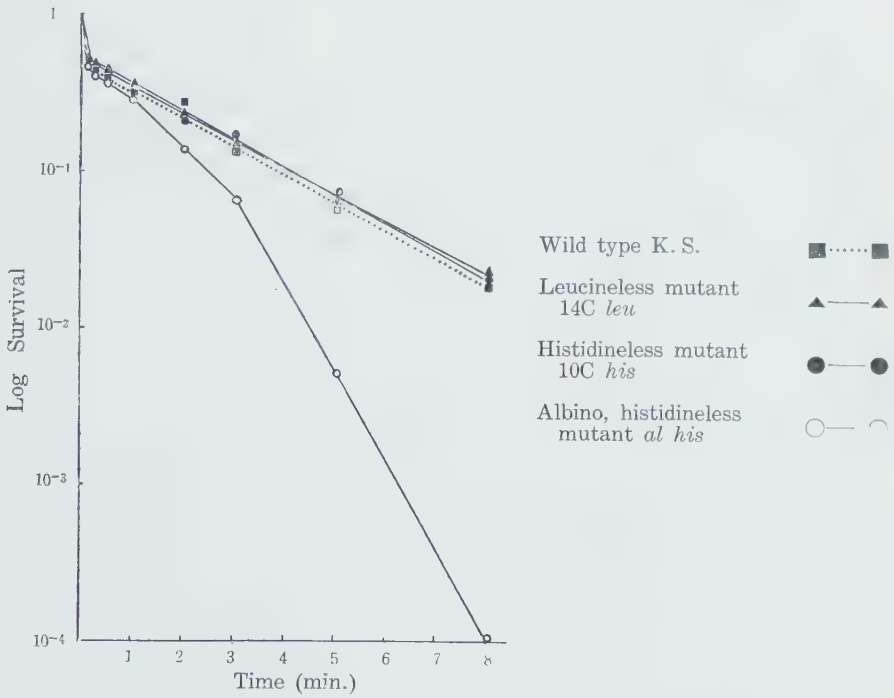


Fig. 1. Survival of the UV-treated Single Auxotrophs of *Asp. sojae*.

the albino strain decreased conspicuously, as compared with the other strains, after five minutes' irradiation.

For the purpose of isolation of mutants, conidia irradiated for 5 or 8 minutes were used with the exception of the albino strain which was irradiated for 3 or 5 minutes. After such treatment, 1 to 5 per cent of the conidia survived.

(4) Isolation Methods

To obtain auxotrophs in filamentous fungi, total isolation and selection methods, including the "filtration method" and the "starvation method",

have been used by previous workers.

i) Total isolation—This original method was developed by the pioneer work of Beadle and Tatum[3] for isolating biochemical mutants *Neurospora*. The conidia irradiated as mentioned above were plated on C. M. After appropriate incubation each colony was picked onto C. M. slants, and then tested on M. M., on which only the wild types (prototrophs) can grow. By this test auxotrophs (biochemical mutants or nutritionally deficient mutants), being unable to grow on M. M. and being able to grow C. M., were isolated. This technique has been extensively used by a great many workers. However, it has proved to be very laborious.

ii) Selection techniques—It was obviously desirable to develop a technique for eliminating the larger population of non-mutant conidia (prototrophs) from the irradiated conidia. Such a technique has been invented by Fries for *Ophiostoma*[4-5]. This method results in a concentration of the mutants. In minimal medium the mutated conidia remain ungerminated or develop only short hyphae, while the non-mutated conidia germinate rapidly and develop small mycelia. Most of the latter are removed by sifting the culture through a suitable filter, and most of the former are found in the filtrate together with all the conidia killed by the irradiation.

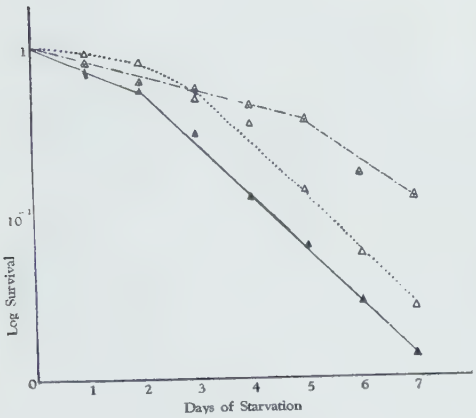
A filtration method has been developed for *Neurospora* as an adaptation of the concentration method devised by Fries; it has been improved by Lein *et al*[6], Woodward *et al*[7-8], and Catcheside[9]. It consists of removing non-mutated strains by continuous filtration of a suspension of irradiated conidia that were allowed to germinate on a medium that would support growth of the wild but not of mutant strains. By this technique biochemical mutants have been screened with apparently high frequency. However, a considerable decrease in the number of mutant conidia occurred, possibly for the following reasons: 1) mechanical elimination of non-germinated conidia, including conidia killed, or having their germination delayed, by irradiation; 2) formation of heterocaryons; 3) a rapid loss in viability of spores kept in liquid medium for prolonged periods.

A successful method has been devised by Lein *et al*[56], based on a microscopic selection of ascospores that do not germinate on M. M.. By this procedure 80 to 95 per cent of the wild type spores can be discarded without further testing.

Starvation technique: The concentration method was effectively modified by Macdonald and Pontecorvo[10], and applied to isolating double auxotrophs of *Asp. nidulans*. The starvatian technique, first so-called by them, was based on the differential survival of single-and double-auxotrophs under starvation conditions in which no supplements are added to M. M..

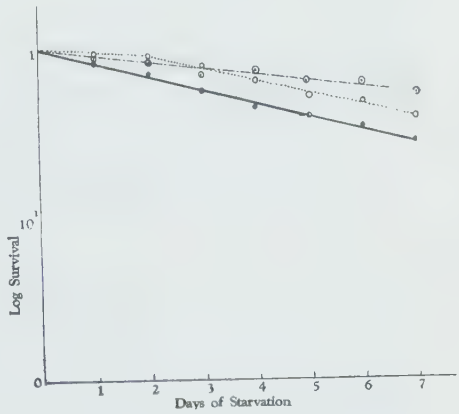
The present authors adopted the total isolation method and the starvation technique for isolating double auxotrophs. In the former case, irraditated condia were immediately plated out on C. M. In the latter case, preliminary tests were made to determine whether the starvation technique would be useful. These tests were as follows: Ultraviolet-treated and

non-treated auxotrophic conidia were plated onto M. M., and starved for 7 days. During the starvation, at intervals of 24 hours, those plates were enriched by the addition of Koji-extract agar on the surface in layer 0.2–0.3 cm. deep. It was recognized that there was a difference in survival between the two types of conidia: the survival ratio of the treated conidia is higher than that of the non-treated conidia (Figs. 2-3). This result suggests that there may be some double auxotrophs among the UV-treated conidia. Afterwards a comparison was made between the survival ratios of single-and double-auxotrophs, and a difference was clearly recognized—e.g. after 120 hours' culture in M. M. the survival ratios were 6.5 per cent for



Non-treated Single Auxotroph
14C *leu* ▲——▲
UV-treated Single Auxotroph
14C *leu* △——△
Double Auxotroph
L415 (*y leu nic*) △—·—△

Fig. 2. Survival of a Non-treated Single Auxotroph, a UV-treated Single Auxotroph, and a Double Auxotroph Kept in a Starved Condition.



Non-treated Single Auxotroph
10C *his* ●——●
UV-treated Single Auxotroph
10C *his* ○——○
Double Auxotroph
H123 (C *his arg*) ⊙—·—⊙

Fig. 3. Survival of a Non-treated Single Auxotroph, a UV-treated Single Auxotroph, and a Double Auxotroph Kept in a Starved Condition.

leucineless spores, 38.1 per cent for leucineless nicotinicless, and 49.1 per cent for leucineless argininеless. In another test a comparison was made in the same way of the survival of two different single auxotrophs (leucineless and histidineless) in M. M. There existed a difference between them, as reported by Pontecorvo; after 120 hours' starvation, 39.1 per cent of the conidia of the histidineless strain were still viable, as compared with 6.5 per cent of those of the leucineless strain.

After 48 hours' incubation in the case of the total isolation method, or after 120 hours' starvation followed by layering with C. M. and 48 hours' incubation in the case of the starvation technique, the colonies which ap

Table 1. Double Auxotrophic and Color Mutants Obtained from Single Auxotrophic Mutants of *Asp. sojae* after UV irradiation.

Strains treated	No. of Survivors	No. of Isolates	Mutant Strains obtained			
			Color Mutants		Biochemical Mutants	
			Albino type	Yellow or Brown type	Reduced nitrate req.	Other nutritional req.
14C *leu**	14144	330**	9	4	10	4
		*180***	*0*	*0*	*13*	*1*
10C *his**	13598	403	18	1	6	0
		402	*2*	*1*	*8*	*3*
al his	4998	98		0	3	0
		107		*0*	*3*	*2*

* J. Agr. Chem. Soc. Japan 28, 939 (1954).
C: Conidial type, *al*: albino, *leu*: leucineless, *his*: histidineless.

** The mutants figured with Roman type were isolated by the "Total isolation method" in contrast to the italicized ones which were isolated by the "Starvation technique".

peared were picked onto M. M. (about 20 colonies per plate). As shown in Table 1 the starvation technique was more efficient than the total isolation method for isolating double auxotrophs of the leucineless strain. However, in the histidineless strains both techniques were equally efficient. This seems to indicate that some strains are more suitable than others for the use of the starvation technique.

For isolating single auxotrophs of *Asp. oryzae* K. B. and of *Asp. sojae* Y_1 (yellow mutant), only the total isolation method was employed.

(5) Characterization of Mutants:

Any auxotroph obtained by the two methods described above cannot grow on M. M., but can grow on C. M. This indicates either a failure to carry out the synthesis of some substances in C. M. and not present in M. M., failure to utilize nitrate as a N-source, or failure to utilize sulphate as a S source. These auxotrophs probably carry mutant alleles of genes necessary for the synthesis of vitamins, amino acids, and other biochemically active compounds.

As a first step, the nutritional requirements of such auxotrophs were determined by observing the growth response on M. M. supplemented with 2 per cent vitamin-free casein hydrolysate, vitamin mixtures, or 2 per cent yeast extract. Growth of any auxotroph on only one of the supplemented plates suggested a further test for individual amino acids, for individual vitamins, and for individual elements of nucleic acids (purines, pyrimidines, and nucleosides).

Secondly, identification of simple growth factors was made by auxanography. This method, used first by Beyerinck in 1889, was applied to the

identification of single and double growth factor requirements of microorganisms by Pontecorvo[11] and Perkins[12]. Heavy suspensions of an auxotrophic strain were plated out on M. M.. Different nutrients possibly required were then simultaneously spotted on the agar surface as crystals or in solution, thus enabling a deficient mutant to grow at those points. This step need not be done with sterile technique because growth response takes place quickly. The great majority of the auxotrophs obtained have turned out to require specific vitamins, amino acids, and purines for normal growth. However, no exact requirements have been determined for the following auxotrophs:

1) Mutants showing no growth on any of the above three meida. Possibly unknown substances are present in Koji-extract agar or the strains require some known factor, but fail to grow on synthetic media because of an inhibitory substance present in too high a concentration (Felix *et al*[13]. Doerman[14]).

2) Strains showing normal growth only on yeast extract agar but no growth on any known elements of nucleic acids tested.

3) Mutants difficult to transfer on test medium because they are sterile types, forming no conidia.

These auxotrophs are listed as "Unknowns".

(5) Stability of Mutants:

For tests of nutritional reversion, conidial suspensions from 7 day-old cultures on Koji-extract agar slants were plated, at a density of 10^6/ml, on M. M. in the case of single mutants, and on M. M. supplemented with the substance required by the original strains in the case of double mutants. The plates were incubated for 5 days, during which time back mutants were found since only they could grow on such media. This test was repeated twice for each strain.

In addition to the reversion of nutritional requirements, the segregation of colony types was also examined. The above conidial suspensions were appropriately diluted and plated onto C. M.; 500–1000 colonies were examined for each mutant.

RESULTS AND DISCUSSION

(1) Types of Mutants:

Table 2. describes the double auxotrophs of *Asp. sojae* obtained by the two different methods among 1520 isolates of different strains. It also includes the single auxotrophs obtained by Iguchi[15]. A glance at the table will show that both types of auxotrophic mutants are very similar except with regards to the requirement for reduced nitrate.

i) Strains requiring reduced nitrate

One of the authors[16] has previously described strains which were able to utilize nitrite but unable to utilize nitrate as a N-source; these were

called "Nitratophobe". These strains are the same as the reduced nitrate mutants found by many workers (Bonner[17]; Beadle and Tatum[18], etc.) and the paraazotrophic mutants of Pontecorvo[10]. Such strains were frequently obtained from various colony types of *Asp. oryzae* in the course of a study of natural variation by the use of successive single spore cultures. M-types generally produced the reduced nitrate types more frequently than did C-types. The reduced nitrate type was also derived from induced mutants of C-and M-type in *Asp. oryzae* aX.

Of 51 double auxotrophs 37 mutants required reduced nitrate, compared with only I out of 37 single auxotrophs. All 37 reduced nitrate strains showed no difference in growth on M. M. or M. M. supplemented with thiosulphate ($Na_2S_2 \cdot 5H_2O$ Ig/l). It is therefore recognized that these mutants do not require reduced sulphur (parathiotrophy, being able to utilize sulphite and being unable to utilize sulphate as sulphur).

As concerns the mutants which are unable to reduce nitrate and sulphate, the first group occurs commonly in *Asp. sojae* and *Penicillium notatum* (Bonner)[17]. On the other hand, the second group is found in *Asp. nidu-*

Table 2. Comparison of Types of Auxotrophs in *Asp. oryzae* and *Asp. sojae*.

Types of Auxotrophs	*A. sojae* (wild type) by IGUCHI	*A. sojae* K. S. (Single Mutant) X-793 (14C *leu*)	X-398 (10C *his*)	H158 (*al his*)	*A. oryzae* 51 (wild type) by IGUCHI	*A. oryzae* K. B. (wild type)
Reduced nitrate	(1)	(23)	(11)	(6)	(8)	(5)
Amino acids	(20)	(5)	(3)	(1)		(7)
arginine	4	1	2		2	1
glycine	1				2	
histidine	3	1			2	1
leucine	3					2
lysine	4	1			4	
methionine	5	2	1			3
multiple						
Vitamins	(7)	(1)		1*		
biotin	1					
nicotinic acid	4					
P. A. B. A.	1					
riboflavin	1					
Nucleic acids Components	(3)					
adenine/hypoxanthine	3	(1)				
Unknowns	(6)	(4)		(1)	(2)	(4)
Totals	(37)	(34)	(14)	(8)	(10)	(16)
No. of Isolates		510	805	205		1329

* Requiring alanine, arginine, glycine, and proline besides histidine.

Table 3. Classification of Koji-molds According to Their Ability to Assimilate N from Various Sources.

Group	N-Source				
	NO_3	NO_2	NH_4	Amino acids	Koji-extract
I	+	+	+	+	+
II	±	+	+	+	+
III	±	±	+	+	+
IV	±	±	±	+	+
V	−	−	±	+	+
VI	−	−	−	±	+

\+ normal growth ± poor growth
− no growth

lans in exceedingly high frequently (331 strains out of 607 mutants, Pontecorvo)[10], and in *Ophiostoma* (74 strains out of 468 mutants, Fries)[19]. Among mutant types selected by the starvation technique, the high proportion of reduced nitrate strains found in *Asp. sojae* seems to be consistent with the remarkable increase of reduced sulphur types found by Pontecorvo in *Asp. nidulans*.

One of the authors[20-23][24] has previously studied nitrite assimilation by fungi. It was found that "Sakaguchi and O's[16] medium", which is supplemented with 1.5 g/l $NaNO_2$ as a N-source (instead of $NaNO_3$ as in Czapek's medium) is useful for the classification of the *Aspergilli*. A new attempt at classification on this basis has been made by using 20 strains of Koji-molds obtained spontaneously or artificially. They were divided into 6 groups as shown in Table 3, according to their ability to assimilate N from various sources. The strains belonging to groups V–VI could not assimilate either nitrite or nitrate. We have been led to suppose that these strains have neither a nitrate nor a nitrite-reducing system[25].

ii) Other biochemical mutant types

The single auxotrophs of two strains of *Asp. oryzae* are also listed in Table 2; some were derived from *Asp. oryzae* 51 by Iguchi *et al*[26], and some from *Asp. oryzae* K. B. by the authors. The production of auxotrophs was undertaken only for the purpose of isolating the genetic materials. In the limited number of isolates so far investigated, however, mutants requiring arginine, histidine, lysine, and methionine have been found most frequently among the three strains of Koji-molds examined. These mutants, except for the histidine auxotroph, were common in *P. notatum* (Bonner), *Asp. nidulans* (Pontecorvo), *Neurospora* (Tatum *et al*[27].), and *Ophiostoma* (Fries)[19]. Tatum states that the types of mutants obtained depend to great extent on the stability of the particular gene concerned.

In *Asp. nidulans* a histidine auxotroph was not obtained by Pontecorvo even among 1346 isolates examined in experiments using selective techniques.

However, in all three strains of Koji-molds, this auxotroph occurred in rather high frequency. The mutant type is common in *Penicillium*, but is non-exsistent in *Ophiostoma*. The glycine auxotroph, which has been found only in *Neurospora*, was isolated in two strains of Koji-molds. Nicotinic acid auxotrophs were found frequently among vitamin requirers ; and among nucleic acid component requirers, adenine or hypoxanthine auxotrophs were common.

Adenine-or hypoxanthine-requring strains have been subdivided into two classes, one normal in color and the other having a purple pigmentation. Three strains (X-750, X-1026, and M-205) belong to the first class, and strain L-387 to the second. These two classes correspond to at least two of the genetic types described previously by Mitchell and Houlahan[28] and by Tatum *et al*[27]. Among nicotinic acid-requring strains strain L 415 also produced a reddish brown pigment in the medium, though other strains with the same requirement (U-432, M-51, M-718, and M-729) did not. Bonner[29-30] supposed that such pigments represent either abnormal metabolism or spontaneous decomposition of intermediate metabolites and probably represent abnormal utilization of an accumulated precursor.

The multiple mutant strain (A139) which was derived from H158 (al his) grows sparsely on alanine, arginine, glycine, or proline, but grows well on a mixture of all four.

Several mutants having the same nutritional requirements can be analyzed genetically to determine whether genes concerned with the same synthesis are allelic or not. The results of our allelism tests will be published in the later section of this work.

(2) Stability of Biochemical Mutants :

Prior to heterocaryon tests, or to the production of supposed heterozygous diploid strains, the nutritional mutants were examined for stability. The major purpose was to determine whether or not there was reversion, which resulted in growth of a nutritional mutant in the absence of an originally required specific substance. Studies dealing with spontaneous reversions of biochemical mutants have been reported by Ryan and Lederberg[31], by Ryan[32-33], and by Giles[34].

i) Single mutants—30 single mutants of *Asp. sojae* were tested: 7 vitamin-requiring strains, 20 amino acid-requiring strains, and 3 base-requiring strains. Among them 4 lysineless, 2 histidineless, and 2 leucineless mutants were comparatively stable in both colony type and nutritional requirement. In the other mutants, however, reversion and segregation of colony types was observed. All the vitamin-requiring strains and most of the methionine-requiring strains were strikingly unstable. These results are largely in agreement with those reported by Giles. He states that reversion occurred spontaneously in most mutants ; however, in two mutants (lysine and pantothenic acid auxotrophs) no reversion appeared either spontaneously or following irradiation. Ryan also indicated that these two

mutants are conspicuously stable among single mutants and that a double leucineless mutant was more stable than either of its component single mutants.

ii) Double mutants—Among double mutants, obtained from the leucineless and the histidineless strains, which are relatively stable single mutants of *Asp. sojae*, 10 (not including reduced nitrate strains) were tested for their stability. All of them proved to be exceedingly stable in colony type and no nutrient-independent back mutants were recovered. Accordingly double mutants are more favorable for recombination studies than are single mutants on account of their high stability and assurance against undetected contamination.

SUMMARY

1. The leucineless and histidineless mutant strains of *Asp. sojae* which proved to be comparatively stable, were irradiated with ultraviolet light, and the following double auxotrophic mutants were isolated by total isolation and starvation techniques.

From strain 10C *his*: requiring reduced nitrate (11 strains), argininelless (2), and methioninelless (1).

From strain 14C *leu*: requiring reduced nitrate (23), argininelless (1), histidineless (1), lysineless (1), methioninelless (2), nicotinic-less (1), and adenine- or hypoxanthineless (1).

2. The starvation technique was effective for isolating double auxotrophs from some strains.

3. Among the mutant types selected by the starvation technique, the reduced nitrate strains were obtained in exceedingly high frequency (37 mutants out of 51 double auxotrophs).

4. In Koji-molds (*Asp. oryzae* and *Asp. sojae*) the most frequent deficient types have been found to be argininelless, histidineless, lysineless, and methioninelless mutants.

5. Among single auxotrophs, lysineless, histidineless, and leucineless mutants were comparatively stable, but other mutants showed frequent spontaneous reversion.

6. Double auxotrophs proved to be more stable than single auxotrophs in such genetic characters as colony type and nutritional requirement.

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IV. CYTOLOGICAL STUDIES

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Previous investigations[1] have shown that the inconstant strains of *Aspergillus oryzae* generally produced large conidia (*Asp. oryzae var. magnaspours*) or sometimes medium sized conidia with occasional large ones (*Asp. oryzae* s. str.), and that the constant strains produced small or sometimes medium sized conidia (*Asp. oryzae var. microsporus*).

The purpose of this paper is to determine cytologically the causes of the difference in variability among these strains. These cytological studies were concerned with the relationship between conidial size and variation, between the number of nuclei in conidia and variation, and with nuclear migration into the conidia of *Asp. oryzae*.

EXPERIMENTAL

1. *Relationship between Conidial Size and Variation.*

Conidial size has already been used as a basis for subdivision in *Asp. oryzae* by one of the authors[2]. However, no work has been done to clarify the relationship between conidial size and variation.

The strains used for this investigation are as follows: Wild strains—C-, M-, and X-colony types obtained after several generations' pedigree culture of three wild strains (*Asp. oryzae* aX, bX, and cX). Six strains were examined of each colony type. Culture strains—*Asp. oryzae s. str.* (6 strains), *Asp. oryzae var. microsporus* (5 strains), and *Asp. oryzae var. magnasporus* (5 strains). Later 17 strains derived from the last named variety were exhaustively studied. In addition, from this variety, homo-type segregants (7 strains) and hetero-type segregants (10 strains) obtained after four generations of pedigree culture were examined.

Cultures were grown on Czapek's agar in petri dishes. The plates were incubated at 30°C for 5 to 7 days. 500–800 conidia from each strain were measured microscopically in dry preparations—without the use of water or any other mounting fluid. Some distribution curves of conidial size are shown in Figs. 1–4.

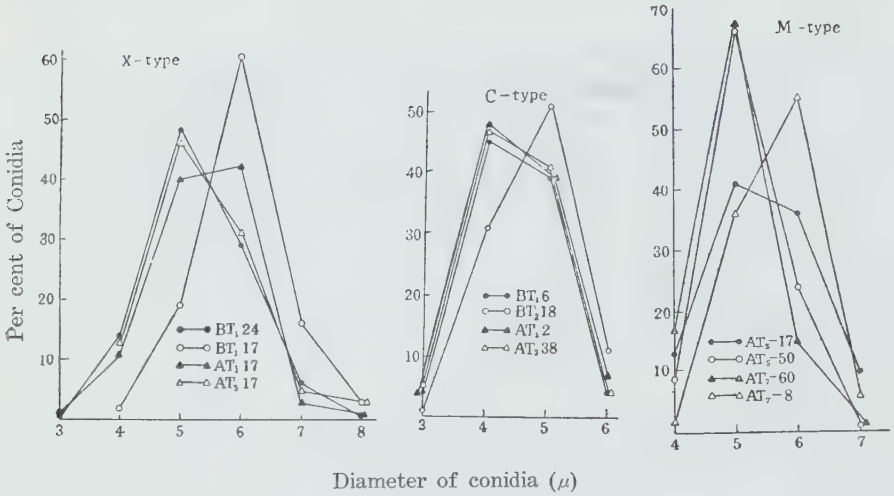


Fig. 1. Distribution of Conidial Size among Single Spore Cultures of Each Variant type from *Asp. oryzae* aX.

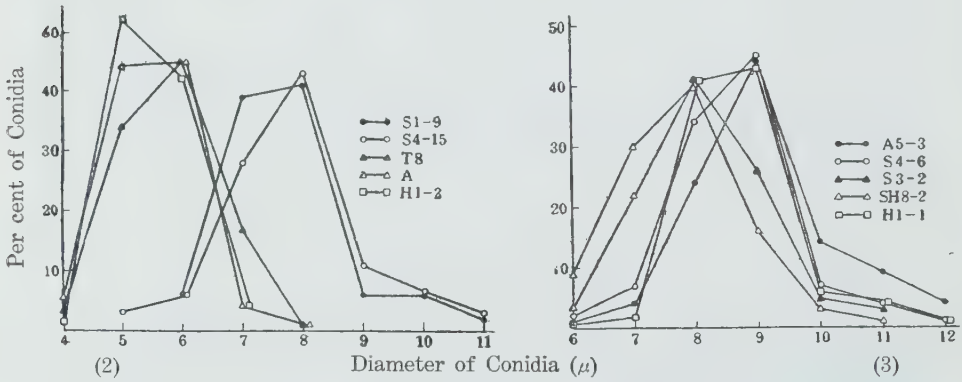
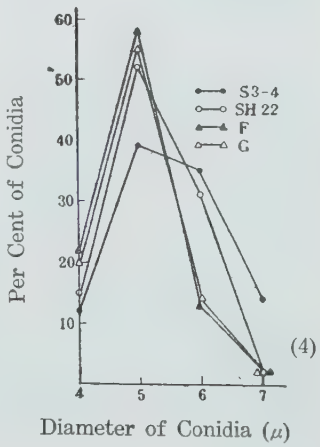


Fig. 2. Distribution of the Size of Conidia of the Each Strain from *Asp. oryzae* s. str.

Fig. 3. Distribution of the Size of Conidia of the Each Strain from *Asp. oryzae var. magnasporus.*

Fig. 4. Distribution of the Size of Conidia of the Each Strain from *Asp. oryzae var. microsporus.*



The constant strains of Koji-molds, C- or M-types, form conidia with comparatively uniform sizes, i.e. C, 3–5 μ and M, 4–6 μ. The inconstant strains, on the other hand, produce conidia in a great variety of sizes including some with extraordinarily large diameters (6–10 μ or more).

Among segregants from the large conidial strains, homo-types form conidia with small diameters (3.5–6.3 μ), while hetero-types form variously sized large conidia (5–10.5 μ).

Attempts at obtaining polyploid nuclei in the conidia have been made by using polyploidogenic agents. The results concerning this problem will be described in the later section of this series.

2. Relationship between Number of Nuclei and Variation.

Previous studies have been made relating to the nuclei of the conidia of *the Aspergilli*. Dangeard[3] reported on *A. fumigatus*, *A. nidulans*, *A. ochraceus*, *A. niger A. flavus*, and *A. herbariorum*. He showed all except the last-named species to be uni-nucleate. Fraser and Chambers[4] dealt with *A. herbariorm*, and Dale[5] with *A. repens*. They indicated that these species had multinucleate conidia at maturiy. Wakayama[6] examined thirteen *Aspergillus* species (not including *A. oryzae* and *A. sojae*). He confirmed the fact that the sterigmata were always uni nucleate. He also showed that the process of mitosis went on regularly according to the well-known scheme of somatic mitosis in higher organisms. Baker[7] investigated *A. fumigatus*, *A. clavatus*, *A. repens*, and *A. echinulatus*. The first three species were described as uninucleate, and the last as multinucleate. The most extensive report of nuclear counts is Yuill's[8] investigation of about sixty species among fourteen groups described by Thom and Raper in their *Manual of the Aspergilli*[9]. He reported that only uninucleate species were found in ten groups—*A. niger* and *A. glaucus*—and only multinucleate species in the *A. tamarii* and *A. flavus-oryzae* groups. Almost all the reports mentioned above consider only the number of nuclei in conidia, without genetic analysis. These reports are correct in stating that there is a distinct difference among species in the number of nuclei in conidia. However, the nuclear number in the same species is differently reported by various investigators. Our studies will contribute to the understanding of the possible causes of this discrepancy.

The majority of cytological studies on filamentous fungi have been carried out with paraffin sections stained by Heidenhain's iron-alum hematoxylin method. More recently a few other staining methods have been used for smear preparations: Newton's crystal violet method (Yuill)[8], aceto-carmine or hematoxylin in propionic acid (Raper and Fennell)[10], aceto-lactic-orcein stain (McClintock)[11], and azure A in $NaHSO_4$ and hydrochloric acid (Huebschman)[12]. There is a danger in that these basic dyes generally respond both to DNA in the nuclear material and RNA in the cytoplasm. Therefore previous treatment with 1 N hydrochloric acid was used in order to remove RNA. After hydrolysis, the following stain-

ing techniques were tested: 1) Rafalko's[13] modification of the Feulgen technique; 2) Robinow's[14] technique; 3) the aldehyde-mordanted basic fuchsin technique of Delamater[15-16]. Heidenhain's method was also compared with the three methods mentioned above.

The cytological pictures obtained by each method have been remarkably consistent, though the degree of staining, and of fading, was variable, decreasing in this order: Delamater's, Rafalko's, Heidenhain's and Robinow's. Hence the first two methods have been extensively employed in our cytological examinations.

The Feulgen technique was first invented by Feulgen and Rossenbeck[17] in 1924 for the demonstration of DNA in tissues. They state that the staining is specific for the potentical aldehyde group of DNA and that after hydrolysis this group remains fixed instead of diffusing through the cell. The presumed specificity of the Feulgen reaction for DNA, which is the chief element in a nucleus, has been verified by a great many workers (Dodson[18], Carr[19], Stowell[20-21]). From a strictly chemical point of view the works of Rafalko[13], Delamater[22], and Winge[23]) on yeast indicate that with proper adjustment of the Feulgen technique no staining of intracytoplasmic or intravacuolar granules is obtained. The present authors propose, therefore, that all Feulgen-positive bodies studied by this technique are nuclei. As additonal evidence, Feulgen-positive mitotic-like pictures were frequently observed in the germ-tubes of conidia or in the spore-forming tubes of sterigmata.

Prior to staining, the following treatments gave satisfactory results for the smear preparations of Koji-molds. Conidia were spot-inoculated on Czapek's or Koji-extract agar in petri dishes at 30°C. When the culture had reached the spore-forming stage, the samples to be stained were put directly on slides with Meyer's adhesive. The rest of the sequence is:

1) Fixation in Carnoy's fluid, 10 min.

2) Washing in 95 per cent alcohol, 3 changes at 2 min. intervals.

3) Hydrolysis in 1 N HCl at room temperature (20°C) for 5 min., at 60°C (in a water bath) for 8 to 10 min., and at room temperature again for 5 min.

4) Washing in distilled water, 5 min.

Proper hydrolysis of the materials in hydrochloric acid is absolutely necessary to get clear and precise cytological picures. Of the various temperatures and operation times examined, acid hydrolysis at 60°C was proved to be optimal at 8–10 minutes. Young conidia with thin walls are the best material for staining. These are usually found after 30 to 48 hours of incubation in plate cultures. After the conidia mature their walls become conspicuously thicker by deposition of chitin, and therefore, in such spores only the conidial wall is stained.

Hydrolyzed materials were stained with the following staining techniques. Rafalko's modification of the Feulgen technique: This method differs essentially from the standard technique only in the manner of preparing the

staining solution and the rinsing bath. By gentle bubbling of SO_2 from a small aperture in glass tubing into 100 ml. of 0.5 per cent basic fuchsin solution, decolorization takes place in about half an hour, and the reagent is then ready for use. Distilled water is similarly saturated for the sulphrous acid rinsing bath. Both leuco-basic fuchsin and sulphurous acid may be stored at 0°C for 1–2 weeks in a dark bottle with a tight cork. SO_2 gas can be easily produced by a simple flask and funnel generator using $Na_2S_2O_5$ and dilute sulphuric acid. The preparations were exposed to the leuco basic fuchsin for one hour, then rinsed in sulphurous acid for 2–3 minutes (sufficient time to remove the free leuco-basic fuchsin), and finally in distilled water for 10–15 minutes. The staining clearly showed not only reddish purple, densely staining Feulgen-positive granules in the conidia, but also Feulgen-positive threads in the germ-tubes. Rafalko's method of decolorization gave much more satisfactory results than the original technique (basic fuchsin decolized with 0.5 g. of Na_2SO_3 or $K_2S_2O_5$ + 10 ml. 1 N HCl).

Some precautions were required in order to eliminate occasional failure in the use of the Feulgen method: 1) The correct timing and temperature of hydrolysis; overhydrolysis should be avoided especially. 2) The use of pure samples of basic fuchsin; poor samples failed to give completely decorized solutions, and sometimes they stained the nuclear structure weakly. The authors used large crystals of the Merck product "Diamant". 3) Leuco-basic fuchsin must be freshly prepared, since SO_2 is the desired active chemical.

Delamater's technique is as follows: A solution of 2 per cent formaldehyde as a mordant was applied to the cells and allowed to act for 2 minutes, after which time the cells were washed briefly in distilled water. By this treatment the nuclei could be intensly stained, and became resistant to decolorization. Following the formalin mordant, the cells were stained in a 0.5 per cent aqueous solution of basic fuchsin for 30 minutes, and again throughly washed in distilled water. This method stained the cytoplasm weakly. By passing through 1/100 N HCl the cytoplasmic stain was removed, while the nuclei remained bright reddish purple.

Robinow's technique is as follows: After hydrolyis the materials were stained with Giemsa's reagent (one drop of the original solution af this reagent was added to a mixture of 1 ml. Söresen's buffer solution at pH 6.8, and 9 ml. boiled distilled water) for 30 minutes. Preparations were rinsed in a solution, made by adding 0.5 ml. of 0.1 per cent K_2CO_3, to 10 ml. distilled water, after both hydrolysis and staining. This technique stained the nuclei reddish purple or bluish purple. Rapidness of diffusion of the dye throughout the preparation was a potential source of trouble.

Heidenhain's Hematoxylin stain is as follows: The process used was exactly the same as Wakayama's. Small portions of colonies on agar plates were cut out and fixed with Allen's modification of Bouin's formalin-picric-glacial-acetic-acid for 24 hours. These were embedded in paraffin and

sectioned 3 to 5 μ thick. Slides were mordanted in 4 per cent iron-alum solution for 30 minutes, and stained in 0.5 per cent Hematoxylin solution for 2 hours. As a result of coloration of the cytoplasm this preparation frequently showed muddy or cloudy nuclei, and thus did not give good results for detailed cytological work.

Feulgen-positive bodies are found in a variety of different guises. A large spherical, vesicular structure is found at all growth stages in the

Table 1. Segregation of Variants from Each Original Strain in Relation to the Size of Conidia, Their Source, and the Number of Nuclei They Contain.

Original Strain	Source	Colony Type of the Original Strain	Size of Conidia (μ)	Number of Nuclei	Colony Type of the Offspring								
					C	Al	St	Cm (X)	MC (X)	Mc (X)	M	MR	Nit	N
aX	Soil	X (Cm)	2.5~6.7	1~6	50					15				4
bX		X (Cm)	2.5~6.7	1~6	13			43		4				2
cX		X (Cm)	2.5~6.7	1~6	22			38						3
J-160		C	2.5~5	1~4	90									
E-29		C	2.5~5	1~4	98									
FG		C	2.5~5	1~4	62									
A3-2	Hattcho-miso-Koji	Al	2.5~5	1~4		50								
A3-8		C	2.5~5	1~4	129									
A-24-1		C	2.5~5	1~4	58									
A-24-3		C	2.5~5	1~4	60									
A-5		X (Mc)	5.0~8.3	4~8	11				27	45				10
A-88-1		X (Cm)	4.5~7	4~8	33			95						1
H	*Asp.*	M	5.5~7.5	4~6							84			6
A	*oryzae*	M	6 ~8	4~6							87			3
B	s. str.	M	5.5~8.5	4~8							70			10
S4-15		X (Mc)	5.5~7.5	4~6	10		38			60	110			8
S1-2		X (Mc)	5 ~8	4~8					72	44				
S7		X (Cm)	5 ~7	4~6				64			8			1
S4-18		X (Cm)	5 ~7.5	4~8				48			8			2
K. B.		C	4.5~6.5	2~4	70									
SH22	*Asp.*	C	4 ~6	1~4	90									1
SH15-3	*oryzae*	C	4 ~5.5	1~4	42									1
S3-4	*var. mic-*	C	4 ~6	1~4	54		1							1
F	*rosporus*	M	4 ~6	1~4							48			2
G		M	4.2~6	1~4							83			3
A5-3	*Asp.*	X (MC)	6~10.5	4~16			1		37	55		1		48
A1-5	*oryzae*	X (MCR)	6~11	4~10					35			30		13
S4-6	*var. ma-*	MR	6~15	6~20							52	40		17
S3-2	*gnasporus*	X (Cm)	7~11	8~20			1	43						2
S2-3		X (Cm)	6~9.2	8~20	58		1	2						
SH8-2		X (Mc)	6~9.5	8~20					4	56			4	3
SH8-4		X (Cm)	7~11	8~10	2		4	84				2		
SH31		X (MC)	6~15.5	8~20	44				27					8
SH34		X (Cm)	6~9	6~16	2			45						
T21		X (Cm)	6~11.2	8~20	74			58						3

S—Saké Koji-mold
SH—Soy Sauce K. M.
T—Tamari-Soy Sauce K. M.
H—Fermented Soybean Paste K. M. (Hattcho)
M—Soybean Paste K. M. (ordinary)
A—Rice mash K.M.

strains tested, but most frequently in mature conidia or sterigmata. On close examination a well-recognizable membrane is observed. It is most clearly visible in the resting cell. Mitotic-like pictures of Feulgen-positive bodies in division, which are thready or chromosomal in appearence, were often found during active growth, i.e. in germ-tubes, hyphal tips, spore-producing tubes, etc. The resolution of the light microscope, however, is not sufficient to permit further detailed observation owing to the minute size of the stainable bodies.

The results obtained by the examination of the number of conidial nuclei in various strains of Koji-molds are given in Table 1. The conidia of the constant strains have been found to contain 1–4 nuclei; about 95 per cent of the condia contained two nuclei. The conidia of the inconstant straines, however, are multinucleate (8–20 or more) (Fig. 5). Among the segregants from 13 strains of *Asp. oryzae var. magnasporus*, homo-types have 2–4 nuclei, while hetero-types have 4–16 nuclei as shown in Table 2.

Discussion—Asp. oryzae had been already subdivided by one of the authors into three varieties according to the size of the conidia.

Group A, with conidia less than 6 μ (*Asp. oryzae var. microsporus*)

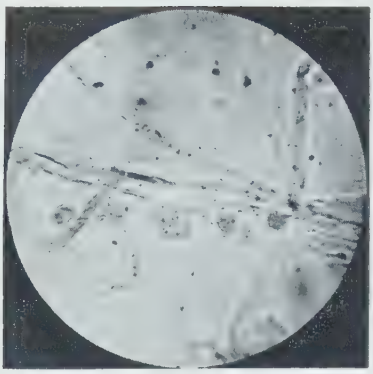
Group B, with conidia 6–8 μ (*Asp. oryzae s. str.*)

Group C, with conidia more than 8 μ (*Asp. oryzae var. magnasporus*)

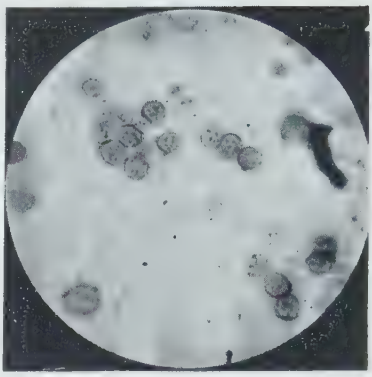
Table 2. Variation of Homo-and Hetero-Type Segregants from 13 Strains of *Asp. oryzae var. magnasporus* in Relation to the Size of Conidia and the Number of Nuclei They Contain.

	Type of Segregant	C_0	C_3	C_5	Al	CR	C_2mm	C_4mm	C_5mm	MCR	MCR	Mc	M	St	MR	N	Diameter of Conidia (μ)	Number of Nuclei
Homo-Type	C_0	+				(+)										+	3.5~5	2~4
	Al				+											+	3.5~5	2~4
	CR					+										+	3.5~5	2~4
	MCII									+				+		+	4.2~5.6	1~4
	St													+		+	3.5~6.3	1~4
	M												+			+	5 ~5.6	1~4
	MR														+	+	3.5~6.3	2~4
Hetero-Type	C_3		+	+												+	5 ~13	4~10
	C_5	+		+					+				+	+		+	4.2~9.8	4~10
	C_2mm	+		+			+	+	+							+	3.5~7	2~6
	C_4mm	+						+	+	+	+	+				+	6.3~9.1	4~8
	C_5mm	+		+			+		+		+	+		+		+	7 ~8.4	4~10
	MCI		+						+	+						+	5.6~9	4~16
	MCIR	+							+		+					+	5 ~10	4~10
	Mc											+			+	+	7 ~10	6~8
	M	+	+	+			+	+		+			+		+	+	6.3~10	4~10
	MR	+						+	+		+			+	+	+	7 ~10.5	4~16

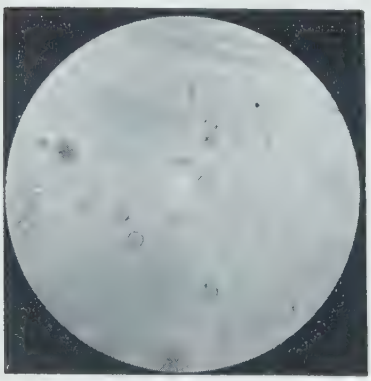
Photograph 1 Nuclei of Conidia in Koji-molds.



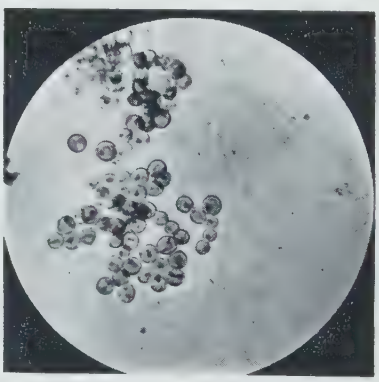
Asp. oryzae var. magnasporus SH31



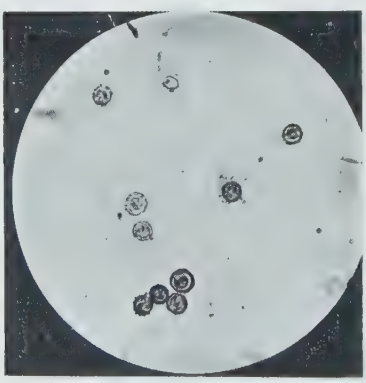
Asp. oryzae var. magnasporus A5–3



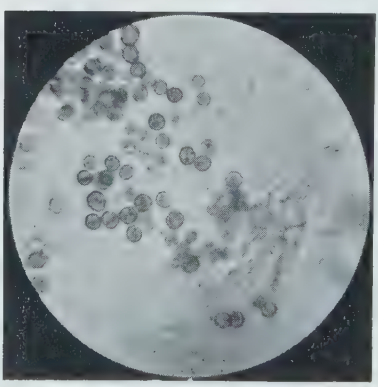
Asp. oryzae s. str. S4 15



Asp. oryzae s. str. K. B.



Asp. sojae SH10–3



Asp. oryzae var. microsporus SH15–3

The present cytological studies establish with certainty that there are clear differecnces in nuclear number among the conidia of these varieties: The number of conidial nuclei is respectively 1–4 in Group A, 4–6 in Group B, and 8–20 or more in Group C. Yuill states that there are two sections in the *Asp. glaucus* and *Asp. niger* groups, that is, small-spored and large-spored sections—spores of the first are usually uninucleate, and of the second, multinucleate. Therefore, if the size of the conidia and the number of their nuclei are used as a basis for subdivisions, the same two sections emerge quite naturally.

The difference in nuclear number of the *Asp. glaucus* group observed by Fraser and Chambers and by Wakayama, seems to be based on taxonomic confusion. Unfortunately there is now no means of determining the type of *Asp. glaucus* Link, since no ascospore descriptions are extant in either report.

It is stated by Yuill that the conidia of *Asp. oryzae* Cohn and of *Asp. flavus* Link contain 1–3 unclei. This condition is found only in our small conidial strains (*Asp. oryzae var.* microsporus). All members of the large conidial strains examined in this study had much larger numbers of nuclei (8–20 or more) than any multinucleate species described in previous report. (e.g. *Asp. echinulate*, having the greatest number of nuclei and large ascospores, contains only 4–8 nuclei according to Baker, and 5–12 nuclei according to Yuill).

The present study further revealed that there is a close relationship between conidial size and the number of nuclei, i.e. the number of nuclei in conidia increase in proportion to the size of the conidia. The situation seems to parallel exactly that described by Yuill. Species with spores which average less than 4μ are usually uninucleate, while those with spores having an average diameter of more than 5μ are substantially multinucleate.

From the facts shown it might be assumed that variability of the inconstant strains of Koji-molds depends principally upon the multinucleate character of the conidia. The next subsection will deal with our observations on this possibility.

3. Nuclear Migration into the Conidia.

There exsists a considerable literature on the number of nuclei in conidia, but little work on nuclear migration seems to have been undertaken except by Baker[7] and Yuill[8].

Cytological studies have been made to clarify whether there is a difference in the nuclear behavior between the constant strains and the inconstant strains. The passage of nuclei from the vesicle to the sterigmata and thence to the condia was observed in detail. Several points of interest have come to light during the course of these experiments.

In this study 4 strains of *Asp. oryzae* were employed: C- and M-type

constant strains of *Asp. oryzae* aX, and the inconstant strains, *Asp. oryzae var. magnasporus* A5–3, and SH–4.

These strains were grown on Koji-extract agar in petri dishes, fixed on slides at intervals of 6, 12, 24, 30, and 48 hours, and stained by the methods mentioned previously.

The conidia of C- and M-type strains are typically binucleate when they are first formed. Germination of the conidia occurs within 6–7 hours after incubation. During the process of germination enlargement of the conidia is conspicuous; they often double their size. In the C-type strain the conidia remain binucleate, while in the M-type strains, they usually become quadrinuclerte through a (probably mitotic) division of each nucleus. In both strains one nucleus moves into the germ-tube. The nuclei which are left in the conidia do not divide unless a second germ tube is formed. The wall of the germ-tube is very thin and delicate; therefore it is easy to stain the nuclei at this stage.

Pictures of vigorously dividing nuclei are clearly observed in elongating germ-tubes. As the hyphae grow rapidly the more distal portions are distinctly cut off with cross walls. The cells between these septa vary widely in length and in number of nuclei. There is no constant positional arrangement of nuclei except that actively growing hyphal tips contain greater numbers. The foot cell, which is differentiated from certain cells in the mycelium, is larger than the adjacent assimilative hyphae, and usually gives rise to one conidiophore by budding and subsequent elongation. There seems to be no way of knowing whether the several nuclei in an initial conidiophore originated from one or more nuclei, because the nuclei are already in division as they move into it from the foot cell. The developing conidiophore is accompanied by many actively dividing nuclei, and is much larger toward the apex than at the point of organ. At maturity further dilation occurs more or less abruptly to produce the vesicle. The vesicle is globose or hemi-spherical, and multinucleate. In the early development of primary sterigmata, which are closely covered with a simultaneously developing layer of cells on the surface, no nuclear migration was observed. When the cells reach their characteristic mature size and shape, one nucleus moves from the vesicle into the sterigma, and divides once in the center of the sterigma. Hence the sterigmata are generally binucleate. About this time a swelling appears at the apex of the sterigma which will become a spore. A narrow spore-producing tube connects it with the sterigma. One of the two nuclei in the sterigma passes into this tube. When the sterigmata are about to produce the conidia, mitotic-like divisions were often clearly observed. Wakayama also showed that the method of nuclear division at this stage is mitosis, and the haploid chromosome number is 2. The spore-producing tube continues to enlarge rapidly, successively cutting off new sections and pushing the older cells outward. Each conidial chain consists of equal sections, and each carries a daughter nucleus derived directily from the successive divisions of

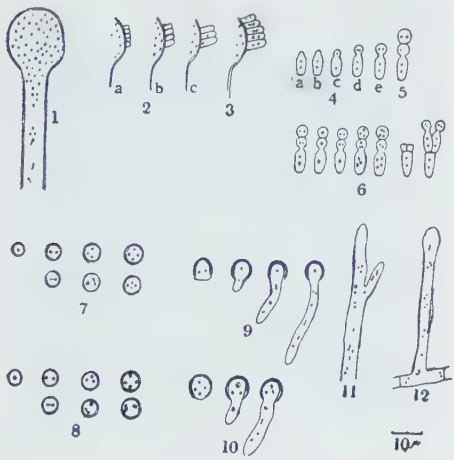


Fig. 6. Cytology of *Asp. oryzae* aX (C-type and M-type).

1. Mature vesicle.
2. Stages in sterigmata development.
3. Mature sterigmata.
4. Stages in conidial development.
5. Normal nuclei in the sterigmata and young conidia.
6. A series of sterigmata and conidia showing the distribution of nuclei.
7. Mature conidia (C-type).
8. 〃 (M-type).
9. Germinating conidia (C-type).
10. 〃 (M-type).
11. Hyphae.
12. Conidiophore and foot-cell.

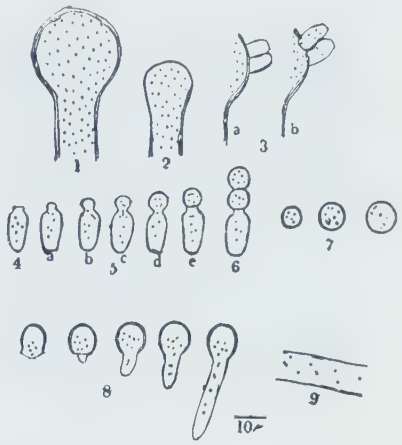


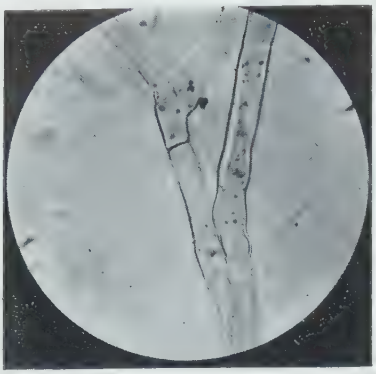
Fig. 7. Cytology of *Asp. oryzae* var. *magnasporus* A5-3.

1. and 2. Mature vesicle.
3. Stages in sterigmata development.
4. Mature sterigmata.
5. Stages in conidial development.
6. Nuclei in the sterigmata and young conidia.
7. Mature conidia.
8. Stages in conidial germination.
9. Hyphae.

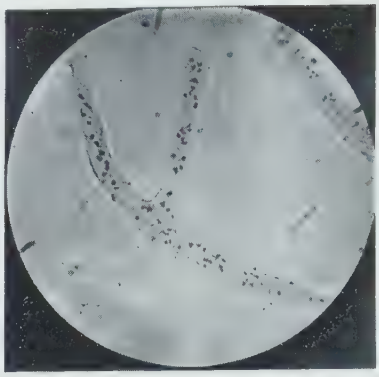
the nucleus in the sterigma. In each conidium, further division occurs immediately, therefore most of the conidia are binucleate maturity. The nuclear migration of C- and M-types of *Asp. oryzae* aX are shown in Figs. 6 and 7.

Asp. oryzae var. magnasporus A5-3 and SH-4, which belong to the large conidial and multinucleate strain, are essentially different from the above two strains in the nuclear migration at the process of sterigmata and conidia formation (Fig. 7). One or more nuclei could be seen at the base of a sterigma, just passing from the vesicle into the sterigma. These sterigmata which are much longer and broader than those in homo-type strains, always have 4-8 nuclei in the central position. A grouping of nuclei or movement of several nuclei from the spore-bearing tube to the conidium was seen just before conidium formation. The conidia become even more multinucleate as a result of independent nuclear divisions occurring during their maturation. At complete maturity there is a rather wide range in diameter of conidia (8-10 μ or more) and in number of

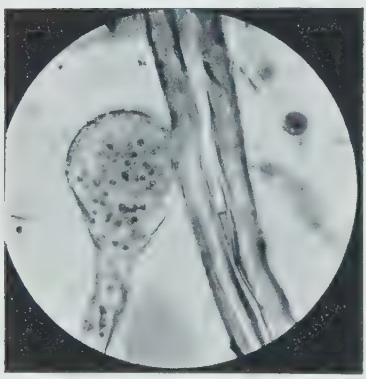
Photograph 2. Cytology of *Asp. oryzae* aX (C-type)



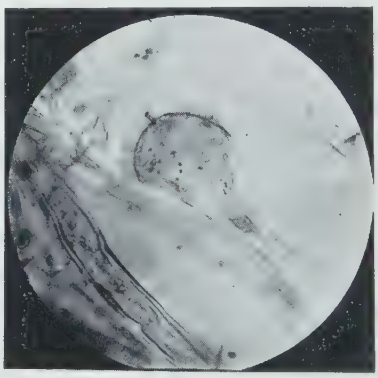
1. Foot-Cell



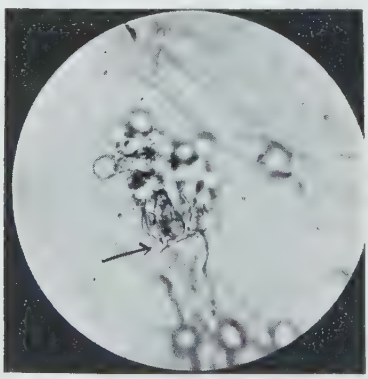
2. Stage in Conidiophore Development



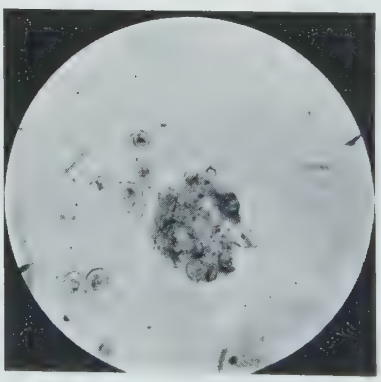
3. Stage in Sterigmata Development



4. Mature Sterigmata



5. Nuclear Migration from Vesicle to Sterigma



6. Nuclei in Conidia

nuclei (8–16 or more). Germinating conidia, which are about twice as large as the original conidia and contain much greater number of nuclei, usually produce 2 or more germ-tubes. Several nuclei move into the large germ-tubes, and continue to multiply there.

Discussion—The observations of the present study show that the nuclei within young conidia divide freely before maturation and germination, and thus a multinucleate condition is secondarily produced. The multimucleate condition in the conidia was established even though the nuclei are derivatives of one original nucleus. Accordingly, the important problem is to determine whether factorial differences may exist in the nuclei entering the sterigmata and the conidia. Baker states that in *Asp. echinulate* the nuclei continue to pass from the vesicle to the sterigmata as conidia are formed. Yuill describes that among multinucleate species in *Asp. carbonarius* the vesicle supplies two nuclei; on the contrary, in *Asp. tamarii* the vesicle may give one nucleus to each sterigma and only as a result of the following division, conidia become multinucleate. The present authors observed that more than two nuclei migrate into the sterigmata and the conidia in the large conidial strains, which produce many segregants. Cytological studies in later experiments confirmed the above results in all 13 strains of the inconstant large-conidial type. Details of nuclear migration in these strains will be described elsewhere. It is accordingly conceivable that variability in the large conidial strains depends upon factorial differences in the conidia as a result of the migration of nuclei of different origins. However, it is most difficult to determine the derivation of the nuclei in the multinucleate condition by cytological survery alone, because no cytological differences between nuclei of dissimilar origines are detected. Genetic evidence is necessary to support this claim. It is quite possible, however, to verify whether or not the vesicle is the primary source of at least two different nuclei in the sterigmata by means of single spore analysis from a heterocaryotic conidial head. As concerns this genetic analysis, the authors have already carried it out by using two color mutants of *Asp. sojae*. Heads with green, yellow, and white conidial chains were developed by the mixed culture of two color mutant strains; a green single conidium from such heads gave rise to a mixed colony-. It might, therefore, be concluded that in this species the vesicle as a rule provides at least two nuclei to each sterigma. Further interesting detailed data on this subject will be presented in the later section of this series.

SUMMARY

1. The causes of the difference in variability among strains of Koji-molds have been studied cytologically by examination of conidial size, number of nuclei in conidia and nuclear migration.

2. The constant strains of Koji-molds have conidia with comparatively uniform sizes (4–6 μ). The inconstant strains, on the other hand, produce

conidia in a variety of sizes including some with extraordinarily large diameters (6–10 μ or more).

3. Feulgen-positive spherical structures are found at all growth stages in strains of Koji-molds. Mitotic-like pictures are frequently observed in germ-tubes, hyphal tips, or spore-producing tubes.

4. The conidia of the constant strains have been found to contain 1–4 nuclei; the majority contained two nuclei. The conidia of the inconstant strains, however, are multinucleate (8–20 or more). It is recognized that there is a close relationship between conidial size and the number of nuclei.

5. In the constant strains, one nucleus moves into conidium from apex of the sterigma at the beginning of the conidium formation, and is divided at once into 2–4 through what is probably mitotic division. In the inconstant strains, more than two nuclei migrate from sterigma into the newly formed conidium. The conidia become further multinucleate (8–20 or more) as a result of prompt independent nuclear divisions.

6. As concerns the migration of the nucleus from the vesicle into the sterigma, the authors have recognized that in the constant strains only one nucleus migrates into the sterigma, while in the inconstant strains at least 2–4 migrate simultaneously.

7. From the facts shown above it might be assumed that the variability of the inconstant strains of Koji-molds depends chiefly upon the multinucleate character of the conidia.

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EFFECT OF VARIATION OF DAY-LENGTH, DAY AND NIGHT-TEMPERATURES, AND INTENSITY OF DAYLIGHT UPON THE GROWTH OF CHLORELLA*

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When *Chlorella* is grown outdoors without artificial regulation of environmental conditions, there occur not only diurnally intermittent variation of light intensity, but also variations of day- and night-lengths accompanied by diurnal changes of temperature. It is now a commonplace that the variations of day- and night-lengths and day- and night-temperatures have manifold effects on the development of higher plants. The study to be reported here was carried out with a view to investigating whether and in what manner such diurnal variations of environmental conditions affect the growth of *Chlorella*.

METHOD

Experimental organism used was *Chlorella ellipsoidea* which was cultured in flat flasks (with a uniform inner thickness of 3 cm) under illumination in the direction perpendicular to the flat surface of the flasks (see Tamiya et al. (1)). To avoid mutual shading of algal cells the population density was kept always below 0.1 ml (packed cell volume) per liter. The culture conditions other than the temperature, light intensity and day-length were kept constant by using a well balanced nutrient solution** and supplying constantly CO_2-enriched air to the culture. The lengths of day and night were varied in the following manner, keeping the diurnal period to be always 24 hours:

Short-day condition: 6 hours light and 18 hours dark,
Equinoctial condition: 12 hours light and 12 hours dark,
Long-day condition: 18 hours light and 6 hours dark.

Cultures under continuous illumination were also run as a control. Day- and night-temperatures studied were 25°, 15° and 7°C, and the intensities of light applied during the daytime were 50, 10, 2 and 0.4 kilolux.

* A paper delivered at the Conference on the Use of Solar Energy, held at Tucson, Arizona, October 31, 1955.

** The culture medium contained, per liter: KNO_3, 5.0 g; KH_2PO_4, 1.25 g; $MgSO_4 \cdot 7H_2O$, 2.5 g; $FeSO_4 \cdot 7H_2O$, 0.003 g; Arnon's A5 solution (2), 1 ml.

The relative growth rate was calculated according to the formula: $k = \frac{1}{t_2 - t_1} \log_{10} \frac{V_2}{V_1}$ where V_1 and V_2 refer to the packed cell volume per liter at the successive times t_1 and t_2 measured in terms of days. Under each specified condition the culture was continued for 10–14 days, and the growth rate was determined by measuring the average daily increase of packed cell volume occurring during the latter 7 to 10 days of the culture.

EFFECT OF CHANGE OF DAY-LENGTH AND DAYLIGHT INTENSITY AT CONSTANT DAY- AND NIGHT-TEMPERATURES

In Figs. 1 and 2 are reproduced the results of experiments, in which the effects of day-length and day-light intensity were investigated at constant day- and night-temperatures.

It may be seen that in the temperature range studied, the growth rate increased markedly with the increase of temperature. At each temperature, the growth rate was directly proportional to the day length at shorter

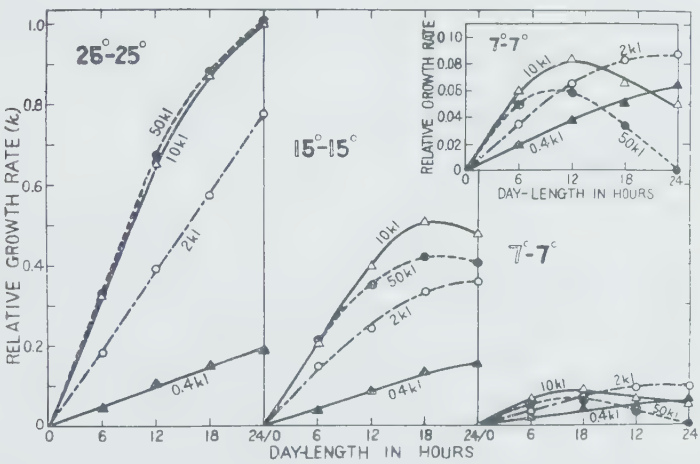

Fig. 1. Growth rate as it changed as a function of day-length and daylight intensity at different temperatures, which were kept the same day and night. The inset shows the results obtained at 7° on a somewhat magnified scale.

day-lengths. Such a proportionality extended to longer day-lengths, the lower the daylight intensity; and the intensity of daylight, under which such a "day-limited" growth occurred markedly, was higher, the higher the temperature. At longer day-lengths, and especially under stronger daylight, the growth rate tended to become independent of day-length or to become rather smaller with the increase of day-length. The lower the temperature, the lower was the intensity of daylight, under which such a

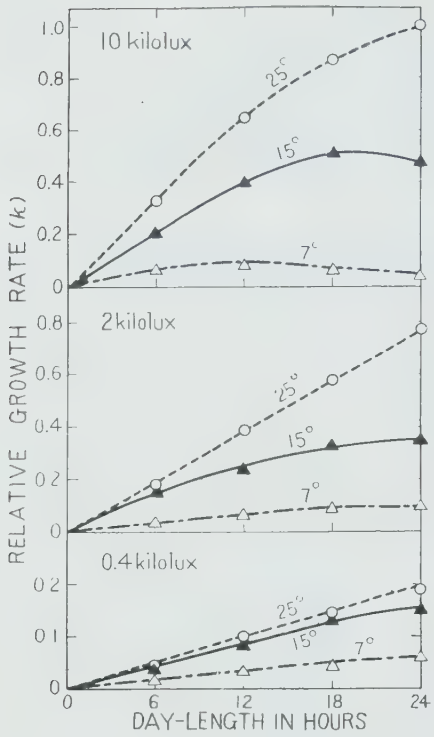

Fig. 2. Growth rate as it changed as a function of day-length and temperature (kept constant day and night) at different intensities of daylight applied.

"day-saturated" or "day-oversaturated" growth occurred, and the shorter was the day-length, at which these phenomena began to take place.

It should be remarked that when growth was "day-oversaturated", more or less strong bleaching of algal cells had taken place.

EFFECT OF CHANGE OF DAY- AND NIGHT-TEMPERATURES

Experiments similar to the above-mentioned ones were performed by changing day- and night-temperatures. In Figs. 3 and 4 are compared the results of experiments, in which night-temperature was the same, and day-temperature was different.

It is apparent that the higher the day-temperature, the greater was the growth rate. It will also be noticed that the higher the day-temperature, the more pronounced was the tendency of day-limited growth, and the less marked was the tendency of day-saturation.

When day-temperature was kept constant and night-temperature was varied, we obtained the results which are summarized in Figs. 5 and 6. Contrary to the profound effect of day-temperature, the night-temperatures affected the growth rate only insignificantly, except in the case given in

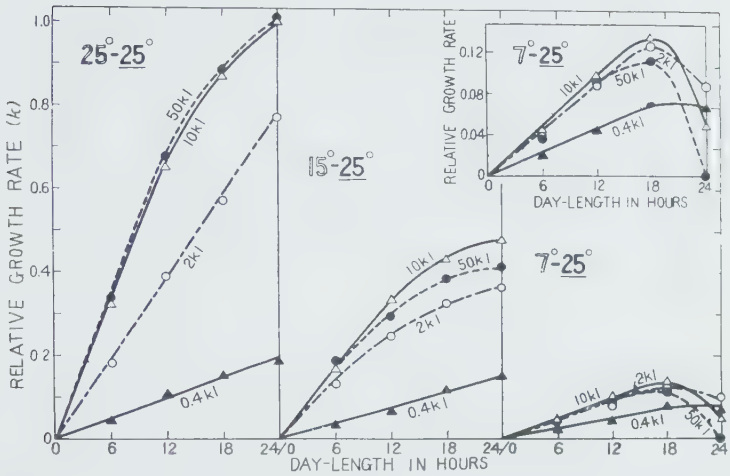

Fig. 3. Growth rate as it changed as a function of day-length and daylight intensity at different day-temperatures (night-temperature being kept the same).

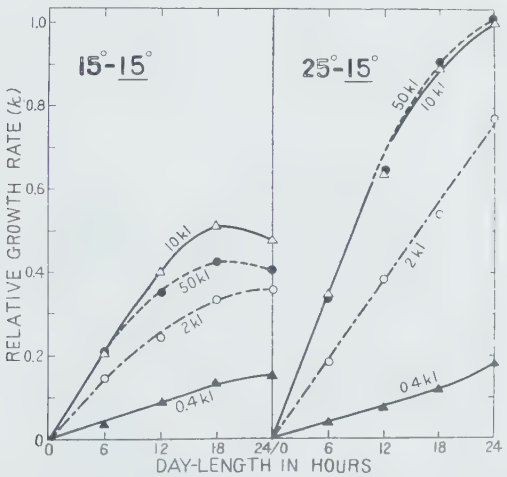

Fig. 4. Growth rate as it changed as a function of day-length and daylight intensity at different day-temperatures (night-temperature being kept the same).

Fig. 6, in which the day-temperature was 7° and the night-temperature was 7° or 25°. When both day- and night-temperatures were 7°, the phenomenon of day-oversaturation occurred markedly at higher light intensities and at longer day-lengths. This was not the case when the night temperature was raised to 25°.

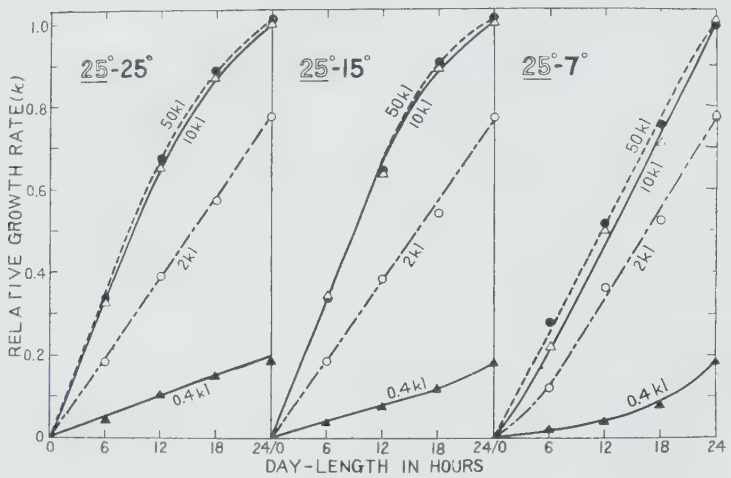

Fig. 5. Growth rate as it changed as a function of day-length and daylight intensity at different night-temperatures (day-temperature being kept the same).

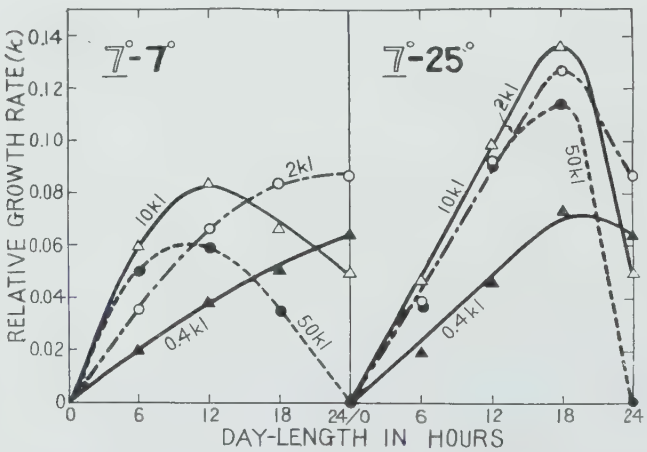

Fig. 6. Growth rate as it changed as a function of day-length and daylight intensity at different night-temperatures (day-temperature being kept the same).

The fact that the day-temperature is more important than the night-temperature in determining the growth rate is also apparent from the curves in Fig. 7, in which the growth rates under equinoctial conditions are plotted against the intensity of daylight.

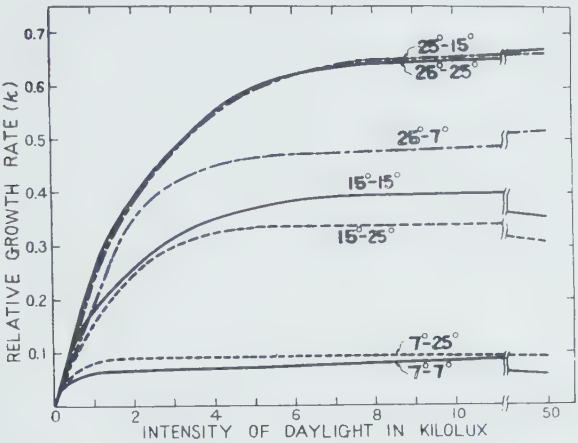

Fig. 7. Growth rate (under equinoctial conditions) as it changed as a function of daylight intensity at different day- and night-temperatures.

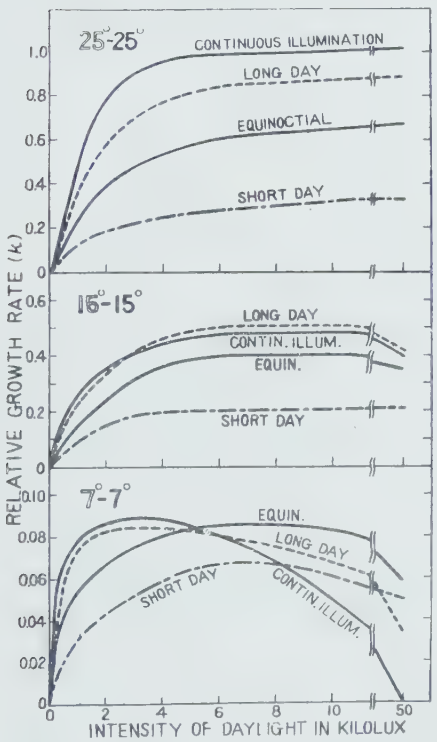

Fig. 8. Relation between growth rate and daylight intensity at different temperatures and different day-lengths. The temperature was kept constant day and night.

RELATION BETWEEN THE DAY-LENGTH, DAYLIGHT INTENSITY AND THE TEMPERATURE-DEPENDENCE OF GROWTH RATE

In Fig. 8 are shown the relationships between the growth rate and daylight intensity as they changed with the day-length and the temperature which was held constant day and night. It will be seen that the difference between the rates under long-day and short-day conditions is more marked at higher temperatures than at lower temperatures; in other words, the advantage of long-day condition over short-day condition decreases with the decrease of temperature. At 7°, and especially when the daylight intensity was high, there was almost no difference in the growth rates under short-day and long-day conditions.

From Fig. 7 showing the reults of equinoctial experiments, it is apparent that the lower the day-temperature, the lower was the day-

light intensity, at which the growth rate became light-saturated. The figure also shows that the temperature-dependence of growth rate decreased and eventually tended to disappear when the daylight intensity was lowered. The same was true at different day-lengths as it is seen from the curves in Fig. 9.

Day-length is also a factor determining the temperature-dependence of growth (see Figs. 9 and 10). At each daylight intensity, the temperature-dependence of growth rate became less and less with the shortening of day-length. These results show that the short-day condition becomes relatively less disadvantageous with the decrease of day-temperature.

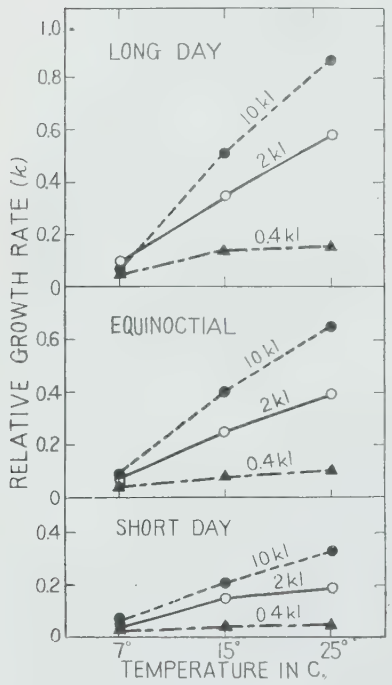

Fig. 9. Temperature-dependence of growth rate at different daylight intensities and different day-lengths. Temperature was kept constant day and night.

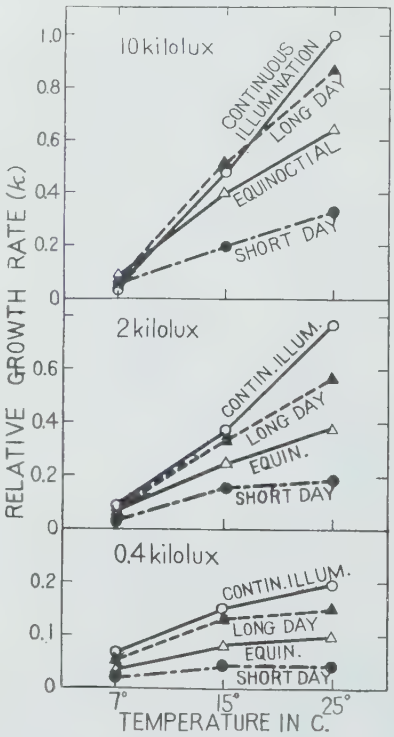

Fig. 10. Temperature-dependence of growth rate at different daylight intensities and different day-lengths. Temperature was kept constant day and night.

DISCUSSION

As we have seen, the growth of *Chlorella* is "day-limited" at shorter day-lengths, under weaker daylight, and at higher temperatures, whereas it tends to be "day-saturated" at longer day-lengths, under stronger day-

light, and at lower temperatures. These situations may be explained on the basis of observations made earlier by Tamiya et al. (3) in their kinetic studies on algal growth. With the idea that the growth process of green plants consists, by and large, of two phases, light-dependent and light-independent processes, they analyzed the relative rates of these two phases under different conditions. They found that the light-independent phase has a considerably greater temperature-coefficient than the light-dependent phase. At higher temperatures, therefore, the light-independent phase will proceed faster than the light-dependent one, and a reciprocal relation will hold at lower temperatures, so that according to the temperature, different phases will become rate-determining for the over-all growth process.

It was also shown by Tamiya et al. (3) that the cells of *Chlorella* assume, in their life cycle, two distinct forms possessing widely different characters. The form, which was named "light cells" is much larger in size, poorer in chlorophyll content, and less photosynthetically active than the other form which was called "dark cells". The change of dark cells into light cells is a light-dependent process, while the formation of (a number of) dark cells from light cells is a light-independent process. For this reason and because of the difference of temperature coefficient of the two phases mentioned above, light cells tend to accumulate to a larger extent, the higher the light intensity and the lower the temperature. It has been observed in other experiments not reproduced here that, when the photosynthetically less active light cells were exposed to strong light, they were sooner or later bleached and, in extreme cases, eventually killed. Conceivably, this is the cause of the phenomenon of "day-oversaturation" which occurred more markedly under higher daylight intensities, at longer day-lengths and at lower temperatures.

It was observed that the day-oversaturation occurred most profoundly when both the day- and night-temperatures were 7°, whereas it did not occur when the night-temperature was raised to 25° (Fig. 6). When both the day- and night-temperature were low, the light cells, which had been formed from dark cells during the cold day-time, were not able to divide during night, whereas at higher night-temperatures they could divide during night to produce dark cells which are not only resistant to strong light but also active in utilizing light to perform photosynthesis. It may be for this reason that the phenomenon of day-oversaturation did not occur at 7° day-temperature followed by 25° night-temperature.

In the physiology of higher plants it has been reported that a moderate lowering of night-temperature as compared with day-temperature has a favorable effect upon growth and development (*Cf.* Went (4)). Such a phenomenon was not observed in *Chlorella* in so far as the temperature range studied was between 7° and 25°C.

SUMMARY

(1) Effect of variation of day-length, day- and night-temperatures and of intensity of daylight upon the growth rate of *Chlorella* was investigated using combinations of conditions which were varied as follows:

Day-length: 6 hours (18 hours dark), 12 hours (12 hours dark), 18 hours (6 hours dark), and 24 hours (no darkness).

Day- and night-temperatures: 25°, 15° and 7°C.

Intensity of daylight: 50, 10, 2, and 0.4 kilolux.

(2) In general the rate of growth was affected by changes of day-temperature by far more profoundly than by changes of night-temperature. In the temperature range studied, the higher the day-temperature, the greater was the growth rate. Higher night-temperatures had no recognizably favorable effect except when the day-temperature was as low as 7°.

(3) In general, the growth rate was directly proportional to the day-length ("day-limited growth") at shorter day-lengths. Such a proportionality extended to longer day-lengths, the lower the daylight intensity; and the intensity of daylight, under which a day-limited growth occurred markedly, was higher, the higher the day-temperature. At longer day-lengths, and especially under stronger daylight, the growth rate tended to become independent of day-length (the phenomenon of "day-saturation"), or to become rather smaller with the increase of day-length (the phenomenon of "day-oversaturation"). The latter phenomenon was accompanied by a bleaching of algal cells. Both the day-saturation and -oversaturation occurred *ceteris paribus* more markedly at lower day-temperatures. The day-oversaturation occurred most profoundly when both the day and night temperatures were 7°, but ceased to occur when the day-temperature was 7° and the night-temperature was 25°.

(4) Although the growth rate increased, in general, with the increase of day-length, the favorable effect of long-day condition decreased with the decrease of day-temperature. At 7°, and especially when the daylight intensity was high, there was almost no difference in the growth rates under short-day and long-day conditions. It was also observed that the temperature-dependence of growth rate decreased and eventually tended to disappear with the decrease of daylight intensity and with the shortening of day-length, and that the daylight intensity, under which the growth rate became light-saturated, was lower at lower day-temperatures. All these facts indicate that the short-day condition and weaker daylight become relatively less disadvantageous with the decrease of day-temperature.

(5) The phenomena of day-limited and day-saturated growths as they were conditioned by temperature, intensity of daylight and day-length were explained on the basis of observations made earlier by Tamiya et al., who, by determining the relative rates of light-dependent and light-independent phases in the growth process of algae, have found that the light-

independent phase has a considerably greater temperature-coefficient than the light-dependent phase.

(6) The phenomenon of "day-oversaturation", which occurred at lower temperatures and under stronger daylight, was explained as being due to the injurious effect of strong light upon the so-called "light cells" which have been shown by Tamiya et al. to become abundant in cultures when temperature was low and light was strong.

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ON α-KETOGLUTARIC ACID FERMENTATION

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In spite of its significant rôle in Krebs TCA cycle, little is known concerning the accumulation of α-ketoglutaric acid as an intermediate product of the bacterial oxidation of glucose. In 1946, the first research was carried out by Lockwood and Stodola.[1] They found that this acid is produced when the 2-ketogluconic acid fermentation of *Pseudomonas fluorescens* NRRL B-6 proceeds beyond the maximum accumulation, and at the point where all the 2-ketogluconic acid has disappeared, about 25% α-ketoglutaric acid is present for initial glucose supplied. More recently, Koepsell, Stodola, and Sharpe[2] have reported a higher yield, about 41% for glucose supplied, in shaken cultures of *Pseudomonas*. Masuo *et al.*[3] confirmed the accumulation of this acid as well as of small amounts of pyruvic- and oxalacetic acids in a strain of *Pseudomonas*, and Katagiri, Tochikura, and Imai[4] have also found that a considerable amount of this acid is produced by a strain of the *Escherichia coli* group. Most recently, Masuo and Wakizaka[5] isolated a new bacterium which gives a high yield of α-ketoglutaric acid, and in many experiments a yield of 50~60% of the acid for initial glucose added was obtained in shaken cultures. This bacterium has been identified as a member of the genus *Bacterium* according to the classification of *Bergey's Manual of Determinative Bacteriology*, (6th edition)[6] and has been named *Bacteriam α-ketoglutaricum* n. sp.

These discoveries are of considerable interest, not only for the industrial production of α-ketoglutaric acid, but also for the study of the mechanism of the bacterial oxidation of glucose.

The present report is concerned with the accumulation of α-ketoglutaric acid from glucose among a variety of aerobic and facultative anaerobic bacteria. A discussion of the mechanism of α-ketoglutaric acid formation is also given. Besides *Pseudomonas* and *Escherichia coli*, *Serratia marcescens* No. 18, *Bacillus megatherium*, *Bacillus natto* (a variant of *Bacillus subtilis*), *Bacterium succinicum*[7], and *Gluconoacetobacter cerinum*[8] were found to produce a considerable amount of α-ketoglutaric acid during the fermentation of glucose in shaken culture.

These bacteria, especially *Serratia marcescens* No. 18 which seemed to have some value for the industrial production of α-ketoglutaric acid, were investigated for the effect of environment on acid accumulation. A new bacterium, isolated from soil and thought to be very similar in its properties to *Bacterium α-ketoglutaricum* Masuo and Wakizaka, was used for the detailed

study of culture conditions favoring the accumulation of α-ketoglutaric acid. The classification of this new bacterium which was found to produce a high quantity of α-ketoglutaric acid, (55 g or more per 100 g of glucose supplied), will be reported later in this journal.

EXPERIMENTAL

I. On the Production of α-Ketoglutaric Acid by *Pseudomonas* and *Serratia*. *Fermentation Studies.*

Four strains of *Pseudomonas fluorescens*, Nos. 33F, 35F, 41F, and 43F, which were isolated and identified in our laboratory by Yokozawa,[9] one strain of *Pseudomonas fluorescens* NRRL B-6 of the American Type Culture Collection, and thirteen strains of *Serratia marcescens*, from stock cultures of the University of Tokyo, were used. They were routinely grown in a medium composed of 76.4 g glucose, 1 g $(NH_4)_2SO_4$, 0.5 g KH_2PO_4, 0.1 g $MgSO_4 \cdot 7H_2O$ per 1000 ml of distilled water. 2.5% sterile $CaCO_3$ was added separately at the time of inoculation. Cultures were grown in 50 ml of the medium, in 500 ml flasks and incubated at 30°C on a reciprocating shaker at the rate of 116 r.p.m. Three cultures of each strain were grown. After five days' incubation, the culture fluids of *Pseudomonas fluorescens* No. 33F and *Serratia marcescens* No. 18 were filtered off and the filtrates were subjected to analysis. No nickel-dimethylglyoxim precipitates were found in these filtrates after oxidation of a 10 ml sample with $FeCl_3$ in acetic acid and distilled, indicating that no acetoin was present, although its occurrence was reported by Asai, Ikeda and Sasaki[10] in a strain of *Serratia marcescens* grown in stationary culture. The remainder of each filtrate was freed from cations by adding oxalic acid and by passing it through the ion exchange resin, Amberlite IR-120, until the oxalate test for calcium was negative. The liquor was then extracted with ether for 50 hours. The ether insoluble part was neutralized with K_2CO_3 and concentrated *in vacuo*, to which ethanol was added until precipitation occurred. This precipitate, after recrystallization from dilute ethanol, was found to be crystals of K- salt of melting point 152°C. It reduced Fehling's solution. Its methylester, of melting point 172°C., and the quinoxaline derivative, of melting point 200°C., were obtained as crystals. The mixed melting point tests quite agreed with those of 2-ketogluconic acid. Gluconic acid was not detected in the ether insoluble part.

The Separation and Identification of α-Ketoglutaric Acid.

The ether soluble part was neutralized with $CaCO_3$ and $Ca(OH)_2$, and filtered after decolorization with carbon. After being concentrated *in vacuo*, the filtrate deposited fine needle shaped crystals which were filtered off. These crystals, suspended in warm water and acidified with H_2SO_4, were subjected to ether extraction. From the ether extracted part, crystals of free acid were obtained. After recrystallization from water they melted

at 115°~116°C. which coincides with the melting point of α-ketoglutaric acid. For identification, the recrystallized substance was converted into the 2, 4-dinitrophenylhydrazone whose melting point of 222°C. quite agreed with the melting point of that of synthetic α-ketoglutaric acid prepared by the method of Umbreit[11]; in addition, the mixed melting point was not depressed.

We obtained this acid from cultures of *Serratia marcescens* No. 18 and of *Pseudomonas fluorescens* No. 33F. As this seems to be the first report of such a finding, the Ca salt of the acid was subjected to analysis after recrystallization and drying *in vacuo* at 100°C..

Analysis: $C_5H_4O_5Ca$

Calculated	C	32.59	H	2.19	Ca 21.75
Found		31.76		2.84	21.14

It was observed that Ca-α-ketoglutarate crystallized out directly from the culture fluid of *Serratia marcescens* No. 18 after the residual $CaCO_3$ and bacterial cells were removed by centrifugation and the fluid was allowed to stand overnight or for several days in the refrigerator.

The Separation and Identification of Pyruvic Acid.

We have observed the production of pyruvic acid in cultures of *Pseudomonas fluorescens* No. 33F. The separation and identification were carried out as follows. After the Ca-α-ketoglutarate crystals were filtered off from the concentrated filtrate, the mother liquor, which seemed to contain a small amount of Ca-α-ketoglutarate, was acidified with H_2SO_4 and extracted with ether. The extracted part was subjected to vacuum distillation at 10~20 mm of atmospheric pressure, with distilled water added several times.

The distillate thus obtained reduced Fehling's solution and ammoniacal silver nitrate, and gave Simon's color reaction for pyruvic acid. When 2, 4-dinitrophenylhydrazine was added, the distillate gave a reddish-orange precipitate characteristic of pyruvic acid. This precipitate was dissolved in 1 N $NaHCO_3$, then acidified with HCl and reprecipitated. After washing with dilute HCl and cold water, the precipitate was recrystallized from methanol and ethylacetate. This product melted at 218°C., which melting point quite coincided with that of synthetic pyruvic acid 2, 4-dinitrophenylhydrazone, and the mixed melting point was not depressed. The p-nitrophenylhydrazone of this acid was also prepared; it showed a melting point of 220°C., which agrees with that given in the literature for the p-nitrophenylhydrazone of pyruvic acid.

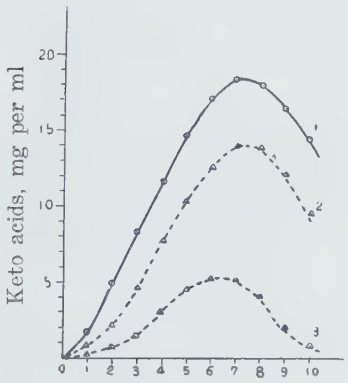
Paper Chromatography of Keto Acids in Fermented Fluid.

A portion of the transparent culture fluid was mixed with 0.7% 2, 4-dinitrophenylhydrazine in 2 N HCl, and a reddish-orange precipitate was formed. The reddish tincture of the precipitate was stronger in the medium from *Serratia marcescens* No. 18. The precipitates from the culture medium of *Pseudomonas fluorescens* No. 33F, were almost completely dissolved by

1 N $NaHCO_3$, while the fluids from *Serratia marcescens* did not give completely soluble precipitates; there remained an undissolved portion of reddish color which was soluble in ethanol. The part of each culture which dissolved in 1 N $NaHCO_3$ was acidified with HCl; precipitates were again formed which were washed with dilute HCl and cold water, thrown into a phosphate buffer of pH 7.2 and again dissolved. Each solution was subjected to paper chromatography [12], using a strip of Tōyō filter paper No. 50 and by the ascending method with a solvent consisting of butanol saturated with 3% ammoniacal water. The culture fluid of *Pseudomonas fluorescens* No. 33F gave two spots, each corresponding with the 2, 4-dinitrophenylhydrazone of α-ketoglutaric acid, R_F value 0.03, and with that of pyruvic acid, R_F value 0.28. On the other hand, the culture fluid of *Serratia marcescens* No. 18 gave only one yellow spot, that corresponding to α-ketoglutaric acid 2, 4-dinitrophenylhydrazone. Therefore it is evident that in the latter strain pyruvic acid accumulation is not detected even by paper chromatography; this fact was also proved in subsequent analytical experiments on the accumulation of keto acids during fermentation.

The Accumulation of α-Ketoglutaric Acid and Pyruvic Acids during Fermentation.

In shaken cultures, *Pseudomonas fluorescens* No. 33F and *Serratia marcescens* No. 18 were observed to produce 2-ketogluconic acid from glucose and also α-ketoglutaric acid when the fermentation proceeded beyond the maximum accumulation of 2-ketogluconic acid. However, α-ketoglutaric acid is not the end product, and it is most likely decarboxylated to succinic acid, as we found succinic acid in the fermented liquor of both strains after 5 days' incubation. This was isolated as crystals of free acid in an ether extract of acidified culture fluid before crystallization of the α-ketoglutaric acid occurred. The melting point was 185°C., which was not depressed by admixture with an authentic specimen of succinic acid.



——: *Serratia marcescens* No. 18
· · · ·: *Pseudomonas fluorescens* 33F
1: α-Ketoglutaric acid
2: α-Ketoglutaric acid
3: Pyruvic acid

Fig. 1. Production of α-ketoglutaric and pyruvic acids by *Ps. fl.* 33F and *S. marcescens* No. 18 in 7.64% glucose containing medium.

In this experiment seven cultures of each strain were grown under the culture conditions described in the section, Fermentation Studies, and at 24 hour intervals one culture of each was subjected to quantitative analysis for α-ketoglutaric, and pyruvic acids. Determinations of both acids were conducted by Shimizu's modified Friedemann-Haugen photometric method [13]. The results are given in Fig. 1.

Table 1. Production of α-ketoglutaric and pyruvic acids in 7.64% glucose containing medium (after 144 hrs' incubation in shaken culture).

Strain	pH	α-Ketoglutaric acid		Pyruvic acid	
		mg/100ml	yield %	mg/100ml	yield %
Ps. fluorescens 33F	5.8	1131	14.80	521	6.82
〃 35F	5.9	1030	13.48	587	7.68
〃 41F	6.2	692	9.06	949	12.42
〃 43F	6.4	1067	13.84	285	3.73
Ps. fluorescens NRRL B-6	6.1	1421	18.60	104	1.36

Seed: Bouillon agar culture

As seen in the figure, α-ketoglutaric acid formation began early in the first 24 hours in both cultures and gradually increased until 168~192 hours, at which time the maximum accumulation was found. The quantity of α-ketoglutaric acid produced in 168 hours incubation was somewhat higher in *Serratia marcescens* No. 18, i.e., 1823 mg per 100 ml, compared with 1384 mg per 100 ml in *Pseudomonas fluorescens* No. 33F. In cultures kept longer than 168 hours the acid gradually disappeared. Pyruvic acid formation was found only in the culture of *Pseudomonas* as previously described, and the maximum accumulation occurred after 144 hours' incubation when 520 mg of acid per 100 ml were produced.

It seems of interest to note that the peak of α-ketoglutaric acid accumulation is preceded by pyruvic acid formation, suggesting that the former might arise from a C_3 compound such as pyruvic acid after the substrate is split into a C_3 compound via 2-ketogluconic acid. Koepsell[14] also stated in his report on gluconate oxidation by *Pseudomonas fluorescens* that α-ketoglutaric acid might be considered to arise during the further oxidation of pyruvic acid, since the apparent transformation of pyruvic acid into α-ketoglutaric acid was observed during the late phases of fermentation.

The Production of α-Ketoglntaric Acid with Five Strains of Pseudomonas and Thirteen Strains of Serratia marcescens.

As previously quoted, Lockwood and Stodola reported a remarkable accumulation of α-ketoglutaric acid in cultures of *Pseudomonas fluorescens* NRRL B-6 grown in a rotary alminum fermenter, and Koepsell, Stodola, and Sharpe reported even higher yields of this acid, i.e., 40.6 g per 100 g of glucose supplied, in shaken cultures of the same organism. In our investigation, *Serratia marcescens* was observed to have greater α-ketoglutaric acid production than *Pseudomonas fluorescens*. An experiment comparing the accumulation of this acid in these two strains was undertaken and the results are shown in Table 1 and 2. Though yields as high as those reported by Koepsell *et al.* in *Pseudomonas fluorescens* NRRL B-6 were not attained in our experiments, we found that *Serratia marcescens* No. 18 is an excellent

Table 2. Production of α-ketoglutaric acid in 7.64% glucose containing medium by *Serratia marcescens* (after 144 hrs' incubation in shaken culture).

Strain		pH	α-Ketoglutaric acid	
			mg/100ml	yield %
Serratia marcescens	I — 2	6.2	1139	14.91
	I — 3	6.5	1207	15.80
	I — 7	6.4	1249	16.35
	I — 9	6.1	1135	14.86
	I —10	6.0	1173	15.35
	II — 1	6.3	1136	14.87
	II — 2	6.8	844	11.05
	II — 3	6.1	979	12.81
	II — 5	6.6	928	12.15
	II — 6	6.3	878	11.49
	II — 7	6.8	1004	13.14
	II —10	6.4	1189	15.56
	No. 18	6.3	1291	16.90
	No. 18*	6.4	1899	24.86

Seed: Bouillon agar culture
* Seed: Koji agar culture

strain for the production of α-ketoglutaric acid; analytical results showed a yield of 24.86 g of the acid per 100 g of glucose in medium containing 7.64% glucose. In other experiments, even higher yields were obtained with *Serratia*, as described below.

II. On the Production of α-Ketoglutaric Acid by *Serratia marcescens* No. 18.

The Formation of α-Ketoglutaric and Pyruvic Acids from Various Carbohydrates.

The basal medium for this experiment was composed of 1.0 g $(NH_4)_2SO_4$, 0.5 g KH_2PO_4, 0.1 g $MgSO_4 \cdot 7H_2O$ per 1000 ml of carbon source-containing distilled water, and sterile $CaCO_3$ in the amount of 25% of the carbon source was added separately. The fermentation was carried out as described in the section, Experimental I. After 7 or 18 days' incubation, a culture fluid was centrifuged at the rate of 7000 r.p.m. for 10 minutes and the supernatant was subjected to determination for dissolved calcium, reducing power and the quantitative analysis of α-ketoglutaric, and pyruvic acids.

As seen in Table 3, α-ketoglutaric acid was formed from all carbon sources tested, whereas pyruvic acid formation could be detected only in the presence of D-fructose. A considerable amount of α-ketoglutaric acid accumulated after 7 days' incubation, especially in medium containing D-glucose, D-mannose, and D-fructose; thereafter the accumulation proceeded slowly. An exception was found in K-2-ketogluconate which could be

Table 3. Production of α-ketoglutaric and pyruvic acids from various carbohydrates.

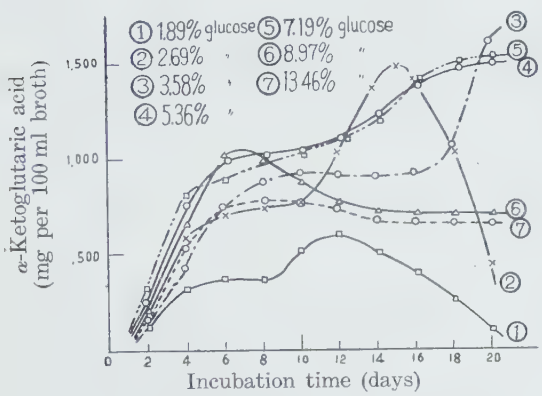
Time	Substrates	D-Glucose	D-Mannose	D-Galactose	D-Fructose	D-Xylose	L-Arabinose	L-Rhamnose	K-gluconate	K-2-keto-gluconate
Initial	pH	6.6	6.6	6.8	6.4	6.5	6.5	6.8	6.9	7.2
	Red. pow.*	14.34	12.09	10.80	11.43	12.22	14.61	7.44	0.22	2.63
	Substrate %	4.58	3.93	3.78	3.92	3.98	3.86	2.52	3.82	1.13
After 7 days	pH	6.6	6.4	6.0	6.2	6.0	6.6	6.5	7.2	8.4
	Red. pow.*	5.80	3.45	6.25	8.50	6.70	0.80	7.34	2.20	0.20
	Ca diss.**	5.2	7.5	1.8	1.6	8.0	9.0	0.5	1.1	0.7
	α-K-G acid mg/100ml Broth.	1070	940	—	730	—	—	—	540	124
	α-K-G acid Yield (%)	23.36	22.92	—	18.62	—	—	—	14.14	10.97
	Pyr. acid mg/100ml Broth.	—	—	—	362	—	—	—	—	—
	Pyr. acid Yield (%)	—	—	—	9.23	—	—	—	—	—
After 18 days	pH	7.4	7.2	7.2	7.5	7.0	6.9	6.8	7.6	8.0
	Red. pow.*	0.09	0.08	5.60	1.15	0.74	1.20	6.94	0.03	0.13
	Ca diss.**	6.1	5.1	3.7	5.5	4.4	4.5	1.1	0.7	0.7
	α-K-G acid mg/100ml Broth.	1460	892	305	946	490	730	201	862	—
	α-K-G acid Yield (%)	31.88	22.68	8.07	24.13	12.31	18.91	7.98	22.57	—
	Pyr. acid mg/100ml Broth.	—	—	—	2.97	—	—	—	—	—
	Pyr. acid Yield (%)	—	—	—	7.58	—	—	—	—	—

* Reducing power: $N/10$ $Na_2S_2O_3$ ml per ml broth. By LEHMANN-SCHOORL method.
** Ca dissolved: mg per ml broth. α-K-G acid means α-ketoglutaric acid.

metabolized rapidly and thus, in the presence of this substance, the formed α-ketoglutaric acid disappeared after prolonged fermentation. The quantity of acid produced from D-galactose, D-xylose, and L-arabinose was somewhat less than from the above-mentioned carbohydrates, even after prolonged fermentation. The miximum accumulation was found with glucose as the substrate, the yield of acid reaching about 32% for initial glucose supplied after 18 days' fermentation. With regard to the mechanism of α-ketoglutaric acid formation from hexose and pentose, no conclusions may be drawn from this single experiment; however, the fact that no accumulation of pyruvic acid is found during the growth of *Serratia marcescens* suggests that its pathway of α-ketoglutaric acid formation is somewhat different from that of *Pseudomonas fluorescens*, except when fructose is the substrate. More interesting is the accumulation of a considerable amount of pyruvic acid in the presence of fructose, notwithstanding the fact that all other carbohydrates, gluconate and 2-ketogluconate are not capable of giving rise to this acid.

The Influence of Carbohydrate Concentration on the Production of α-Ketoglutaric Acid.

For this experiment, glucose was used as the substrate and the basal medium was composed of 0.1 g $(NH_4)_2SO_4$, 0.05 g KH_2PO_4, and 0.01 g $MgSO_4 \cdot 7H_2O$ in 100 ml of distilled water. Sterile $CaCO_3$, in the amount of 25% of the glucose content, was added separately. Incubation was carried out for 20 days at 30°C. The results are as follows (Fig. 2).


① 1.89% glucose ⑤ 7.19% glucose
② 2.69% " ⑥ 8.97% "
③ 3.58% " ⑦ 13.46% "
④ 5.36% "
α-Ketoglutaric acid (mg per 100 ml broth)
1,500
1,000
500
0 2 4 6 8 10 12 14 16 18 20
Incubation time (days)

The corresponding highest yields: ① 31.64%, ② 54.28%, ③ 44.41%, ④ 27.95%, ⑤ 20.87%, ⑥ 11.59%, ⑦ 5.76%.

Fig. 2. Influence of glucose concentration.

It was observed that the optimal concentration of glucose seemed to lie at about 3~4%, and beyond this, the higher the concentration, the lower the yield. However, the acid yield might be affected by the concentration of nitrogenous substances in the culture medium or by other culture conditions, so we cannot draw conclusions from this one experiment.

The Influence of the Nitrogen Source and its Concentration on the Production of α-Ketoglutaric Acid.

Twelve different nitrogen sources, as listed in Table 4, including $(NH_4)_2SO_4$ and urea, were employed to test the effect of nitrogen source.

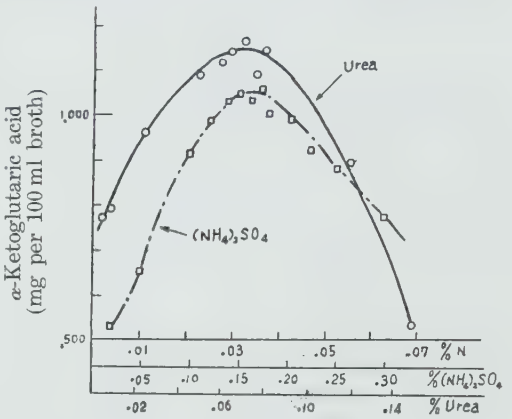
Table 4. Influence of nitrogen sources on the production of α-ketoglutaric acid

No.	N-source	N-comp'd		N-concentration	Yield of α-ketoglutaric acid	
		M.W.	N%	%	mg/100ml Broth	%
1	$(NH_4)_2SO_4$	132.15	21.21	0.10	772	10.63
2	HN_4NO_3	80.05	35.00	0.06	738	10.16
3	$NH_4H_2PO_4$	115.10	12.17	0.17	605	8.35
4	$(NH_4)_2HPO_4$	132.13	21.21	0.10	743	10.24
5	NH_4Cl	53.50	26.17	0.08	441	6.09
6	KNO_3	101.11	13.86	0.15	—	—
7	$NaNO_3$	85.01	16.48	0.13	—	—
8	$NaNO_2$	69.01	20.29	0.10	—	—
9	Polypeptone		12.35	0.17	882	12.15
10	Urea	60.04	46.70	0.05	941	12.96
11	L-Asparagine	132.08	21.21	0.10	1105	15.23
12	Na-Aspartate	173.00	8.09	0.26	1041	14.37

Glucose 7.26%, N-source (see above) corresponding to approximately 0.02% N. Incubation: 144 hrs. in shaken culture at 30°C.

It was found that asparagine and Na-aspartate were excellent nitrogen sources for α-ketoglutaric acid accumulation.

Among the inorganic nitrogen sources, no distinguishable effect on the accumulation was found; in particular the non-assimilability of nitrate and nitrite is interesting, especially when compared with the investigation of Kondō[15] who reported that nitrate is a good nitrogen source for the 2-ketogluconic acid fermentation of *Pseudomonas*.



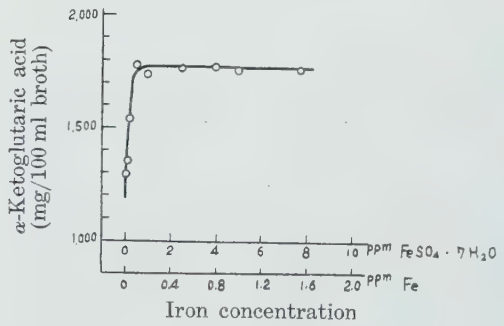
Glucose 7.19%, N-source (see above).
Incubation: 144 hrs. in shaken cnlture at 30°C.

Fig. 3. Influence of nitrogen concentration.

The concentration of the nitrogen sources was more important for acid production than their nature. For instance, when $(NH_4)_2SO_4$ and urea were used, the optimal concentrations were observed to be 0.15~0.17% and 0.06~0.08%, respectively, (corresponding to 0.03% N), and there seemed to be a rather sharp peak at these levels. A remarkable decrease of acid production resulted at concentrations either higher or lower than these (Fig. 3).

The Effect of Iron on the Production of α-Ketoglutaric Acid.

It has been reported by Koepsell[14] that in *Pseudomonas*, the addition to the medium of 0.02 μM/ml of iron as ferrous ammonium sulfate resulted in a marked stimulation of gluconate oxidation. It was considered that the slow rate of oxidation of purified gluconate beyond 2-ketogluconate might be due to a lack of iron for the synthesis of iron-containing respiration catalysis. Therefore we examined the effect of iron on α-ketoglutaric acid accumulation by adding it to cultures of *Serratia marcescens*, first as the ferrous sulfate, later in the form of the ferric salt (Fig. 4, Table 5).



Glucose 4.57%, urea 0.07%.
Incubation: 144 hrs. in shaken culture at 30°C.

Fig. 4. Effect of adding ion.

Our results confirm the report of Koepsell, showing that a trace of iron is essential for acid accumulation; the optimal amount seemed to be

Table 5. Effect of ferrous- and ferric-ions.

No.	Fe-comp'd.	ppm	pH	Red. pow.	Ca diss.	α-Ketoglutaric acid		
						mg/100ml Broth	%	Comparison*
1	$FeSO_4 \cdot 7H_2O$	5.0	6.8	2.04	5.3	1760	38.51	1.52
2	$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	7.0	6.8	1.21	5.3	1780	38.95	1.53
3	$Fe_2(SO_4)_3$	8.0	7.2	1.02	5.2	2240	49.34	1.93
4	$Fe(NH_4)(SO_4)_2 \cdot 12H_2O$	8.6	7.2	1.36	5.4	1720	37.64	1.48
5	—		6.6	2.38	5.9	1160	25.38	1.00

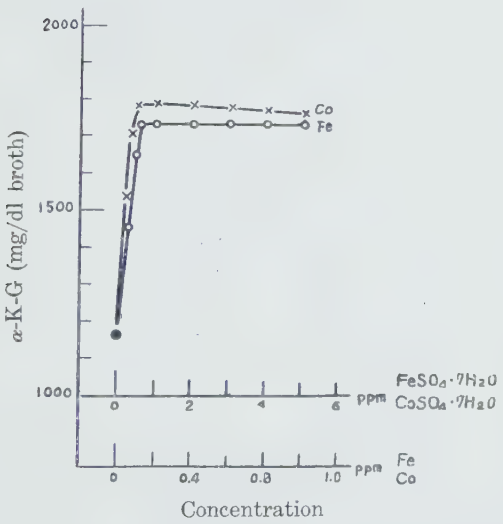
* Comparison shows comparative ratio of yield with No. 5, the standard, no iron added.

Med.: glucose 4.57%, urea 0.07%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.01%, $CaCO_3$ (sterilized separately) 1.25%, and iron (see above).

Incubation: 144 hrs. in shaken culture at 30°C.

about 0.5~8.0 ppm $FeSO_4 \cdot 7H_2O$, corresponding to 0.1~1.6 ppm Fe, when 4.57% of glucose and 0.07% of urea were supplied in the medium. The addition of the ferric instead of the ferrous salt gave similar results, and with either the maximum accumulation of acid was about twice that of the control; we obtained a yield of 49.34%, compared with a control yield of 25.38% for the initial glucose. This suggests that most probably Fe^{++} and Fe^{+++} maintained an equilibrium state between them during fermentation and aided the accumulation of acid.

The Effects of Other Metals on the Production of α-Ketoglutaric Acid.



Incubation: 7 days in shaken culture at 30°C.

Fig. 5. Effects of adding iron and cobalt on α-ketoglutaric acid accumulation.

In this experiment the basal medium was composed of 4.57 g glucose, 0.07 g urea, 0.05 g KH_2PO_4, and 0.01 g $MgSO_4 \cdot 7H_2O$ in 100 ml of distilled water. Sterile $CaCO_3$, in the amount of 25% of the glucose, was added separately. The fermentation was carried out as described in Experimental I. A divalent metallic ion, cobalt, was tested for its effect on α-ketoglutaric acid accumulation. As shown in Fig. 5, its rôle in glucose oxidation was identical with that of iron in the fact of activating the enzymic systems concerned with α-ketoglutaric acid accumulation. This was ascertained by the

Table 6. Effect of metallic ions.

Metallic ions	Comp'ds added	pH	Red. pow.	Ca diss.	α-K-G		
					mg	Yield %	Comparative ratio
control		5.8	2.93	6.87	935	20.7	1.00
Na^+	NaCl 1×6^{-1} M	5.9	2.58	6.07	1280	28.4	1.37
K^+	KCl 1×6^{-1} M	5.8	2.42	5.07	1125	24.9	1.21
Mg^{++}	$MgCl_2\cdot6H_2O$ 0.02μM	6.0	5.03	6.91	597	13.2	0.67
Mn^{++}	$MnSO_4\cdot7H_2O$ 〃	5.9	6.07	7.72	1295	28.6	1.38
Fe^{++}	$FeSO_4\cdot7H_2O$ 〃	5.7	6.10	7.39	1580	35.1	1.69
Co^{++}	$CoSO_4\cdot7H_2O$ 〃	5.7	6.06	7.57	1655	36.6	1.77
Ni^{++}	$NiSO_4\cdot7H_2O$ 〃	5.8	2.86	6.41	1245	27.6	1.34
Cu^{++}	$CuSO_4\cdot5H_2O$ 〃	5.6	5.83	7.23	1440	31.9	1.54
Zn^{++}	$ZnSO_4\cdot7H_2O$ 〃	5.9	4.86	6.34	1085	24.1	1.16
Mo^{VI}	Na_2MoO_4 〃	6.0	2.10	6.17	1050	23.5	1.12
Cd^{++}	$CdSO_4\cdot7H_2O$ 〃	5.6	5.57	7.02	1655	36.6	1.77

Glucose 4.52%, $(NH_4)_2SO_4$ 0.1%.
Reducing power: $N/10$ $Na_2S_2O_3$ ml/ml broth. By LEHMANN-SCHOORL method.
Ca dissolved: mg/ml broth.
α-K-G mg: mg/100 ml broth. 7 days' incubation at 30°.

addition of both iron and cobalt to the culture medium. The effects of other metals are summarized in Table 6. Cobalt, cadmium and iron gave a marked stimulation, but the effects of nickel, copper and manganese were somewhat lower, and the other metals tested had no noticeable influence except magnesium. It is interesting to note, however, that the addition of natrium as NaCl in the rather large amount of 1×6^{-1} M resulted in a considerable stimulation. Subsequent experiments (Table 7) in which 0.1 ~1.0% NaCl was added to the medium gave similar results.

Table 7. Effect of sodium chloride.

NaCl %	pH	Red. power	Ca diss.	α-K-G	
				mg/100ml	Yield %
control	3.7	3.68	4.89	1200	24.4
0.1	3.9	3.18	5.15	1280	26.0
0.2	3.7	3.80	5.25	1340	27.3
0.4	〃	3.69	5.43	〃	〃
0.5	〃	4.24	5.55	1420	28.9
0.6	〃	4.10	5.40	1560	31.7
0.8	〃	4.96	5.38	1400	28.5
1.0	3.9	5.14	5.33	1340	27.3
1.2	4.7	5.38	5.05	1120	22.8
1.4	4.7	5.83	4.89	1050	21.4

Glucose 4.91%, urea 0.07%. Incubation: 7 days at 30°C.

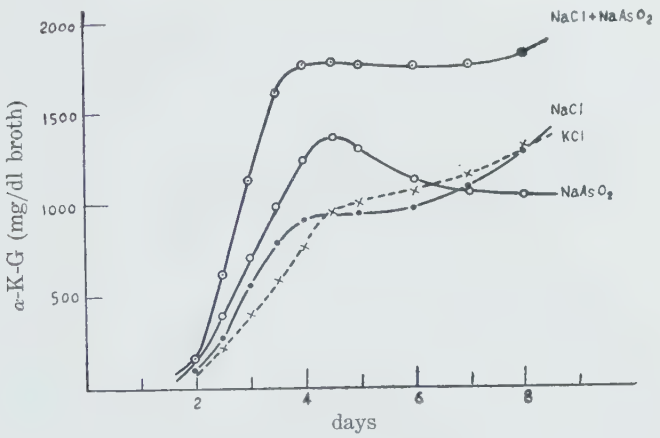
The Effects of Arsenite and an Arsenite-NaCl Mixture on α-Ketoglutaric Acid Production.

Arsenite is known as an inhibitor of the oxidative decarboxylation of α-ketoglutaric acid to succinic acid, and it was reported by Walker, Hall and Hopton[16] and by Hockenhull *et al.*[17] that the accumulation of α-ketoglutaric acid occured in *Aspergillus niger* after the addition of K-arsenite. As seen in Table 8, a stimulative effect appeared when Na-arsenite was added in concentrations of 1×10^{-4} M~3×10^{-3} M, and in this range the acid accumulation was increased by about 20% over the control. Some inhibition was recognized in concentrations of arsenite higher than 1×

Table 8. Effect of sodium arsenite.

$NaAsO_2$	pH	Red. power	Ca diss.	α-K-G	
				mg/100ml	Yield %
control	5.9	5.28	4.75	955	19.4
1×10^{-4} M	5.8	4.16	4.66	1150	23.4
5×10^{-4} 〃	5.9	4.17	4.77	1175	23.8
8×10^{-4} 〃	6.0	3.57	5.44	1100	22.4
1×10^{-3} 〃	5.2	5.50	5.54	1220	24.8
3×10^{-3} 〃	5.0	5.18	4.92	1190	24.3
5×10^{-3} 〃	5.0	6.22	4.84	1020	20.8
8×10^{-3} 〃	4.3	5.39	4.29	980	19.9
1×10^{-2} 〃	3.5	5.32	4.09	700	14.1

Med.: identical with Table 7.
Incubation: 7 days at 30°C.



NaCl 0.6% (0.1 M), KCl 0.75% (0.1 M), $NaAsO_2$ 0.01%.
NaCl+$NaAsO_2$: NaCl 0.6%+$NaAsO_2$ 0.01%.
Med.: identical with Table 7.

Fig. 6. Effect of NaCl, KCl and sodium arsenite.

10^{-2} M, however. Most interesting is the phenomenon of the multiplicable effect of the simultaneous addition of arsenite and NaCl, as shown in Fig. 6.

The Effects of Various Vitamins on α-Ketoglutaric Acid Production.

1 mg of various vitamins was dissolved in 1 dl of sterilized distilled water and passed through a Seitz filter. Each vitamin, in a concentration of 10 γ dl, was added to the culture medium by using this aqueous solution. After 7 days' incubation, quantitative determinations of α-ketoglutaric and pyruvic acids were carried out (Table 9).

Table 9. Effect of added vitamins.

Vitamins	pH	Red. power	Ca diss.	α-K-G (mg/dl)	Pyruvic acid (mg/dl)
Control	5.7	6.58	5.28	835	—
Thiamin	5.7	6.56	5.89	1040	—
Riboflavin	5.8	5.88	3.29	895	20.7
Pyridoxine	6.0	6.72	6.49	1040	trace
Nicotinic acid	5.9	4.97	4.38	1160	—
Nicotinic amide	5.7	5.47	6.11	1050	22
Pantothenic acid	5.8	7.81	4.02	613	192
Folic acid	5.7	7.34	4.25	815	trace
Biotin	5.7	6.79	5.05	955	trace
Choline hydrochloride	5.7	6.79	5.16	910	trace

Med.: glucose 4.72%, $(NH_4)_2SO_4$ 0.1%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.01%, $CaCO_3$ 1.25%.
Incubation: 7 days at 30°C.

This experiment demonstrated that thiamin, pyridoxine, nicotinic acid and nicotinic amide gave a considerable stimulation to α-ketoglutaric acid accumulation, whereas riboflavin, biotin, cholin hydrochloride and folic acid did not show any noticeable effect. In the case of pantothenic acid, however, a remarkable accumulation of pyruvic acid was observed. No clear conclusions may yet be drawn from these results; however it is well known that some vitamins are constituents of coenzymes, and the effects observed may be due to a stimulation of enzymic action resulting in the accumulation of α-ketoglutaric acid.

III. On the Production of α-Ketoglutaric Acid by *Bacillus megatherium*, *Bacillus natto* and *Bacterium succinicum*.

We have found that α-ketoglutaric acid fermentation occurs not only in *Pseudomonas fluorescens* but also in *Serratia marcescens* in shaken culture. The interesting fact is that *Serratia marcescens* metabolizes glucose into acetoin in stationary culture as reported by Asai *et al.*, and the same phenomenon was demonstrated by them in *Bacillus mesentericus*[10]. Therefore we examined the oxidative products formed in shaken cultures with

a substrate of glucose, using *Bacillus mesentericus fuscus*, *Bac. mesentericus vulgatus*, *Bac. megatherium pumilus*, *Bac. megatherium* De Bary, *Bac. subtilis* Hannover strain, two varieties of *Bac. natto* (a variant of *Bacillus subtilis*) and *Bacterium succinicum* Sakaguchi and Tada. Among these strains, we found that *Bac. megatherium* De Bary, *Bac. natto* and *Bact. succinicum* produced a considerable amount of α-ketoglutaric acid as well as a small quantity of pyruvic acid during fermentation, and it was also ascertained by paper chromatography that citric-, malic-, and lactic acids were produced by *Bac. natto*.

The culture medium was composed of 8.64 g glucose, 0.1 g $(NH_4)_2SO_4$, 0.05 g KH_2PO_4, and 0.01 g $MgSO_4 \cdot 7H_2O$ in 100 ml of distilled water to which 2.5 g of sterile $CaCO_3$ was added separately. Cultures of 50 ml of medium in 500 ml flasks were incubated at 30°C. on a reciprocal shaker at the rate of 116 r.p.m. with a stroke of 7 cm. The paper chromatography of keto acids formed during fermentation was carried out by the same procedure as described above, and two spots of the 2, 4-dinitrophenylhydrazones of keto acids were detected in positions corresponding to those of α-ketoglutaric and pyruvic acids.

We therefore separated the keto acids according to the procedure described before, and pyruvic acid was obtained as a derivative of 2, 4-dinitrophenylhydrazone. It melted at 218°C. and this melting point, when the substance was mixed with an authentic specimen of synthetic pyruvic acid, was not depressed.

Analysis: $C_9H_8O_6N_4$ (the preparation from the culture of *Bac. natto* 1)

Calculated	C	40.29	H	3.01
Found		40.45		2.90

The p-nitrophenylhydrazone of this acid, prepared from the distillate of the ether extract of the acidified culture fluid, melted at 220°C., agreeing with the melting point of pyruvic acid given in the literature. The undistilled part of the ether extract of the acidified culture fluid was neutralized with $CaCO_3$ and $Ca(OH)_2$ and concentrated *in vacuo*, after which the rather difficult soluble Ca salt precipitated out. This precipitate, after recrystallization from water, was converted into free acid and subjected to ether extraction. From this extract colorless crystals of melting point 115~116°C. were obtained after recrystallization from water. No melting point depression was observed when they were mixed with synthetic α-ketoglutaric acid. The 2, 4-dinitrophenylhydrazone of this acid showed a melting point of 222°C., just the same as that of α-ketoglutaric acid. The Ca salt and the 2, 4-dinitrophenylhydrazone of this acid, obtained from the culture medium of *Bac. megatherium* De Bary, were subjected to analysis.

Analysis: $C_5H_4O_5Ca$

Calculated	C	32.59	H	2.19	Ca	21.75
Found		32.23		2.92		21.75

$C_{11}H_{10}O_8N_4$

Calculated	N	17.18
Found		17.21

The Accumulation of α-Ketoglutaric Acid and Pyruvic Acids during Fermentation.

The culture conditions and the methods of analysis described above were followed except for the quantitative determination of α-ketoglutaric acid. Because the reddish color developed by the addition of 4N NaOH to the 2, 4-dinitrophenylhydrazone of α-ketoglutaric acid was not stable and a yellow color which appeared in 10% Na_2CO_3 prior to the addition of NaOH was observed to be more stable and more accurate for colorimetric determinations, we adopted the latter procedure. The results are shown

Table 10. Production of keto-acids by *B. megatherium* DE BARY (in shaken culture).

No.	Hrs.	pH	α-Ketoglutaric acid		Pyruvic acid	
			mg/dl	Yield (%)	mg/dl	Yield (%)
1	24	6.8	73	0.84	21	0.24
2	48	6.8	182	2.11	34	0.39
3	72	6.7	371	4.29	67	0.77
4	96	5.8	674	7.81	123	1.42
5	120	6.0	1021	11.82	168	1.94
6	144	7.4	1377	15.85	243	2.82
7	168	8.1	1692	19.58	302	3.48
8	192	6.8	1781	20.62	327	3.78
9	216	6.4	1398	16.36	335	3.87
10	240	6.9	912	10.57	321	3.71

Med.: glucose 8.64%, polypeptone 0.4%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.01%, $CaCO_3$ (sterilized separately) 2.5%.

Table 11. Production of keto-acids by *B. natto* 1 (in shaken culture).

No.	Hrs.	pH	α-Ketoglutaric acid		Pyruvic acid	
			mg/dl	Yield (%)	mg/dl	Yield (%)
1	24	6.9	48	0.55	18	0.20
2	48	6.8	127	1.47	42	0.48
3	72	6.7	289	3.24	98	1.13
4	96	6.5	494	5.72	189	2.18
5	120	7.6	772	8.93	282	3.26
6	144	7.8	1108	12.82	344	3.98
7	168	7.9	1262	14.61	332	3.84
8	192	7.4	1181	13.69	301	3.49
9	216	7.0	939	10.87	269	3.12
10	240	7.2	432	4.99	173	1.88

Med.: see Table 10.

Table 12. Production of keto-acids by *B. megatherium* De Bary (in shaken culture).

No.	Hrs.	pH	α-Ketoglutaric acid		Pyruvic acid	
			mg/dl	Yield (%)	mg/dl	Yield (%)
1	24	6.8	107	1.16	10	0.12
2	48	6.4	187	2.16	13	0.16
3	72	6.2	283	3.27	48	0.56
4	96	6.1	376	4.35	62	0.73
5	120	6.0	541	6.48	96	1.11
6	144	6.3	639	7.39	102	1.17
7	168	6.0	873	10.09	191	2.21
8	192	6.2	1029	11.90	209	2.42
9	216	5.8	1094	12.63	195	2.32
10	240	6.4	1125	12.99	192	2.31

Med.: glucose 8.64%, $(NH_4)_2SO_4$ 0.1%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.01%, $CaCO_3$ (sterilized separately) 2.5%.

Table 13. Production of keto-acids by *B. natto* 1 (in shaken culture).

No.	Hrs.	pH	α-Ketoglutaric acid		Pyruvic acid	
			mg/dl	Yield (%)	mg/dl	Yield (%)
1	24	6.8	—	—	—	—
2	48	6.6	—	—	—	—
3	72	6.4	16	0.19	4	0.04
4	96	6.5	42	0.49	9	0.17
5	120	6.9	95	1.09	21	0.24
6	144	7.0	211	2.44	57	0.65
7	168	6.2	326	3.76	76	0.88
8	192	6.8	406	4.69	108	1.10
9	216	7.6	434	5.02	147	1.69
10	240	6.9	495	5.49	175	2.02

Med.: see Table 12.

in Tables 10, 11, 12 and 13, and the comparative yields from cultures of *Pseudomonas fluorescens* No. 33F and *Serratia marcescens* No. 18 are shown in Table 14. These experiments show that α-ketoglutaric acid formation occured during the first 24 hours and the maximum accumulation was reached in 168–192 hours' incubation with both *Bac. megatherium* De Bary and *Bac. natto* 1 when polypeptone (Takeda) was employed as the nitrogen source. When $(NH_4)_2SO_4$ in approximately the equivalent N content, was used, however, the peak of α-ketoglutaric acid accumulation was reached somewhat later and a lower yield of the acid resulted.

Table 14. Production of keto-acids by various strains tested (in shaken culture).

No.	Strain	α-Ketoglutaric acid		Pyruvic acid	
		mg/dl	Yield (%)	mg/dl	Yield (%)
1	*B. megatherium* DE BARY	1377	15.85	243	2.82
2	*B. natto* 1	1108	12.81	344	3.98
3	〃 2	998	11.51	241	2.78
4	*Bact. succinicum*	1175	13.59	220	2.54
5	*Pseudomonas fluorescens* 33F	1261	16.41	551	6.74
6	*Serratia marcescens* No. 18	1694	22.24	—	—

Med.; No. 1, 2, 3, 4: glucose 8.64%, polypeptone 0.4%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.01%, $CaCO_3$ (sterilized separately) 2.5%.
No. 5, 6: glucose 7.46%, $(NH_4)_2SO_4$ 0.1%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.01%, $CaCO_3$ (sterilized separately) 2.5%.
After 6 days incubation at 30°C.

Pyruvic acid was also detected in 24 hours' incubation with both strains and production gradually increased. However, even at its maximum accumulation the yield was very low compared with that of α-ketoglutaric acid.

Though we make no deductions from these experiments with regard to the mechanism of formation of both acids from glucose, and no evidence is given concerning the existence of gluconate and 2-ketogluconate as intermediates, there seems to be a similarity in the mode of fermentation found here and that of *Pseudomonas*, in which the α-ketoglutaric accumulation is predominant in over-all fermentation. The oxidative pathway is therefore thought to be analogous in these microorganisms.

As shown in Table 14, the yields of α-ketoglutaric acid from cultures of *Bac. megatherium* De Bary, *Bac. natto* and *Bact. succinicum* were observed to be lower than the yield of *Serratia marcescens* No. 18. The maximum yields from these cultures were 20.6% in *Bac. megatherium* De Bary, 14.6% in *Bac. natto* 1, and 13.6% in *Bact. succinicum* for glucose supplied (8.6% glucose in the medium).

IV. On the Production of α-Ketoglutaric Acid by *Gluconoacetobacter cerinum*.

Gluconobacter and *Acetobacter* are known to produce gluconic, and 2-ketogluconic acids from glucose; however the oxidative pathway beyond 2-ketogluconate is little known except for the findings of Katznelson, Tanenbaum and Tatum[18], who reported the formation of 2, 5-diketogluconic acid in a strain of *Acetobacter melanogenum*.

Recently Aida and Asai[19] have found the interesting fact that *Gluconoacetobacter liquefaciens* (*Gluconoacetobacter* is the subgenus of *Gluconobacter*) produces some new γ-pyrone compounds, i.e., Rubiginol (3, 5-dihydroxy-1, 4-pyrone) and Rubiginic acid (3, 5-dihydroxy-1, 4-pyrone-2-carboxylic acid),

from glucose in shaken culture, and it was ascertained that these γ-pyrone compounds are also produced from 2-ketogluconate[20].

However, this strain of *Gluconoacetobacter* seems to be very different in some properties as compared with other species of *Acetobacter* and *Gluconobacter*, although its oxidative behavior is not different in the point of producing gluconate and 2-ketogluconate from glucose. As already stated, we found α-ketoglutaric acid accumulation in many species of aerobic and facultative anaerobic bacteria, viz., *Pseudomonas fluorescens*, *Serratia marcescens*, *Bacterium megatherium*, *Bacillus natto* and *Bacterium succinicum*, in the course of the oxidative fermentation of glucose.

Now it seemed interesting to ascertain whether *Gluconobacter*, a typical aerobic bacteria which accumulates gluconate and 2- or 5-ketogluconate in the course of glucose oxidation, is capable of producing α-ketoglutaric acid as are the above-mentioned microorganisms. An experiment was undertaken to clarify this problem and it was demonstrated that α-ketoglutaric acid, as well as pyruvic acid, are the oxidative metabolic products of glucose, developed via gluconate and 2-ketogluconate.

Fermentation Studies.

Gluconoacetobacter cerinum (isolated by Asai[8]) was used for this experiment. The medium was composed of 0.8% yeast extract water containing approximately 5% glucose. Medium (100 ml in 500 ml flasks) was sterilized for 15 min at 120°C. and sterile $CaCO_3$ in the amount of 25% to glucose supplied, was added separately. Fermentation was carried out at 30°C. on a reciprocating shaker at the rate of 150 r.p.m. The method of analysis was the same as described in the foregoing experiment.

The Separation and Identification of Fermentation Products.

After 4 days' incubation the culture fluid was centrifuged, decationized with oxalic acid and Amberlite IR-120, concentrated *in vacuo* and subjected to ether extraction. The procedure is shown in Table 15. Fraction (A) (K salt) was obtained as prismatic crystals. It was decolorized with carbon and recrystallized from dilute ethanol. The crystals showed a melting point of 152°C. and reduced Fehlings' solution and ammoniacal silver nitrate solution. The methylester showed a melting point of 172°C. A sample of 0.2 g of the K salt was taken and dissolved in 1 ml water to which 0.1 ml concentrated HCl and 0.12 g *o*-phenylendiamine were added; after stirring, the quinoxaline derivative of the acid precipitated out. After recrystallization from hot water, it showed a melting point of 200°C. From the above data, it is quite certain that fraction (A) is 2-ketogluconic acid, and this was confirmed by spectrophotometric analysis of the quinoxaline derivative (using a Beckmann Spectrophotometer, Model DU). The maximum band of the absorption spectrum was observed at 335 mμ (Fig. 7) which quite coincides with the preparation obtained from 2-ketogluconic acid, and with the figure in the literature[21].

Table 15. Separation and identification of acids.

Ether extraction

Soluble fraction
distilled *in vacuo*

distillate
HCl sln. of 2, 4-dinitrophenyl-hydrazine
crystal crude
recrystal. from ethyl acetate
crystal purified (2, 4-dinitrophenylhydra-zone of pyruvic acid)
(C)

residue
neutralized with $CaCO_3$ and $Ca(OH)_2$, concentrated *in vacuo*
crystal (Ca salt of α-K-G*)
recrystal. from water, H_2SO_4 acidic
ether extract
crystal (α-K-G)
HCl soln. of 2, 4-dinitrophenyl-hydrazine
crystal crude
recrystal. from ethyl acetate
crystal purified (2, 4-dinitrophenylhydra-zone of α-K-G)
(B)

Insoluble fraction
neutralized with K_2CO_3, concentrated *in vacuo*, EtOH added
crystal crude
recrystal. from EtOH soln.
crystal purified (K-2-ketogluconate)
(A)

* α-K-G means α-ketoglutaric acid.

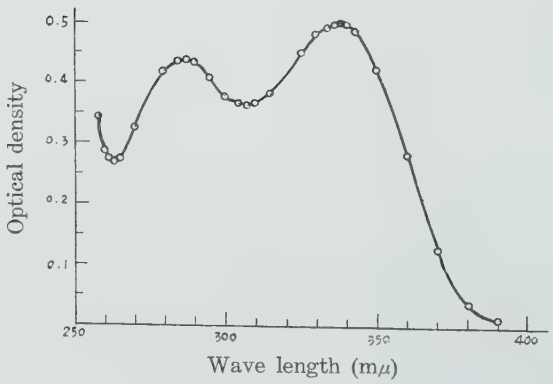


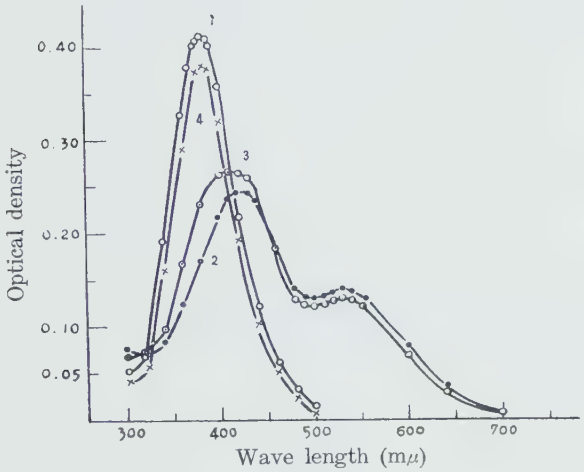
Fig. 7. Absorption spectrum of 2-hydroxytetrahydroxybutyl quinoxaline (quinoxaline derivative of 2-ketogluconic acid) (6.86×10^{-5} M).

Fraction (B) was determined to be α-ketoglutaric acid 2, 4-dinitrophenylhydrazone by its melting point (222°C.), by a mixed melting point test with an authentic specimen, and by its elemental analysis.

Analysis: $C_{11}H_{10}O_8N_4$

Calculated	C	40.00	H	3.09	N 17.18
Found		40.47		2.96	16.71

A spectrophotometric analysis was also carried out, the results of which are shown in Fig. 8 and Table 16. The figures obtained quite agree with those in the literature[21] and with that of the synthetic acid.



1: in 10% Na_2CO_3
2: in 1.25 N NaOH
3: in 10% Na_2CO_3 and 1.25 N NaOH mixture
4: in M/100 phosphate buffer (pH 7.2)

Fig. 8. Absorption spectra of 2, 4-dinitrophenylhydrazone of α-ketoglutaric acid.

Table 16. Maximum absorption band of 2, 4-dinitrophenylhydrazone of α-ketoglutaric acid.

Solvents	Standard (mμ)	Experimental (mμ)
(a) 10% Na_2CO_3	380~383	381
(b) 1.25 N NaOH	424~426	424
(c) 10% Na_2CO_3+1.25 N NaOH	405~407	406
(d) M/100 phosphate buffer	380~382	381

Fraction (C) was determined to be pyruvic acid 2, 4-dinitrophenylhydrazone by its melting point (218°C.), by a mixed melting point test with an authentic specimen, and by its elemental analysis.

Analysis: $C_9H_8O_6N_4$

Calculated	C	40.29	H	3.01	N 20.90
Found		40.03		2.78	21.28

Moreover, the absorption spectrum quite agreed with that of the synthetic acid and with figures given in the literature[22] (Fig. 9 and Table 17).

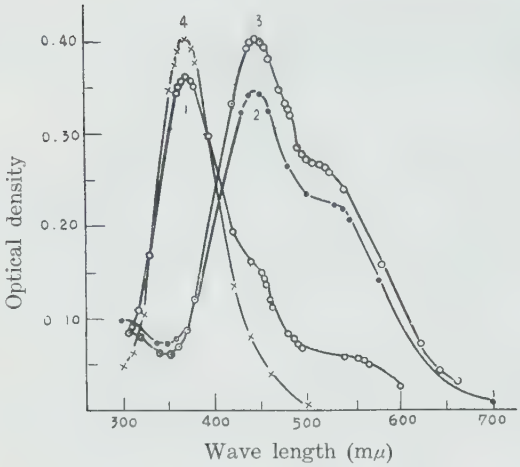


Fig. 9. Absorption spectra of 2, 4-dinitrophenylhydrazone of pyruvic acid.

Table 17. Maximum absorption band of 2, 4-dinitrophenylhydrazone of pyruvic acid.

Solvents	Standard (mμ)	Experimental (mμ)
(a) Na_2CO_3	370~372	371
(b) 1.25 N NaOH	444~446	444
(c) 10% Na_2CO_3+1.25 N NaOH	445~447	445
(d) M/100 phosphate buffer	370	370

Chemical Changes during the Fermentation of Glucose.

During 7 days' incubation, the change of pH, the amount of dissolved Ca, the reducing power and the quantities of α-ketoglutaric, and pyruvic acids were traced and determined. As seen in Fig. 10, the pH decreased in the initial stages accompanied by a rapid consumption of glucose and acid formation; thereafter it gradually increased to a stationary stage, while dissolved Ca and acid accumulation were both observed to reach a maximum after about 2 days' incubation. At this time the reducing power and the pH were found to be at their lowest. The rapid decrease of reducing power, accompanied by the rapid increase of dissolved Ca in the initial stages of fermentation, suggests that gluconate production occurs early and rapidly; thereafter gradual decomposition into pyruvate and α-ketoglutarate may take place, and it seems most likely that α-ketoglutarate is produced via pyruvate because the peak of accumulation of pyruvate appears earlier than that of α-ketoglutarate.

These phenomena were observed in our experiments with *Pseudomonas*, and we suppose that the oxidative pathway of glucose in *Gluconoacetobacter*

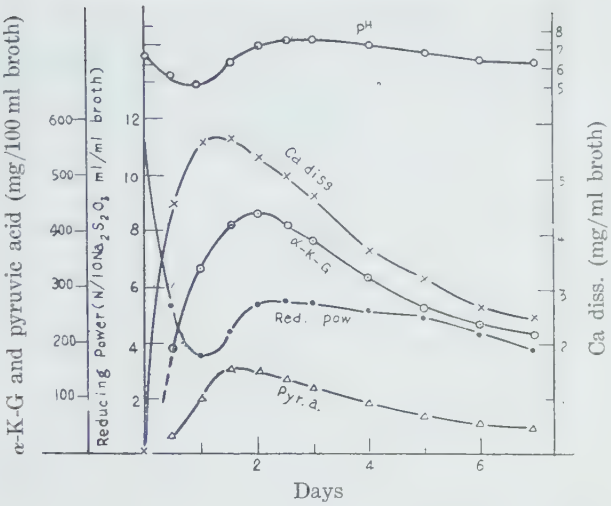


Fig. 10. Chemical changes during the fermentation of glucose.

Table 18. Formation of α-ketoglutarate and pyruvate from glucose, gluconate and 2-ketogluconate.

			Gluconoacetobacter cerinum			*Serratia marcescens* No. 18		
			Substrates					
R_F values			Glucose	Ca-gluconate	K-2-keto-gluconate	Glucose	Ca-gluconate	K-2-keto-gluconate
Free acid	pyruvic	0.73	+	+				
	α-ketoglutaric	0.56	+	+	+	+	+	+
	malic	0.32				+		
2, 4-Dinitrophenylhydrazone	pyruvic	0.46	+	+				
	α-ketoglutaric	0.05	+	+	+	+	+	+
pH			5.8	5.6	7.2	5.8	5.9	8.9
Red. power*			1.74	0.03	0.08	4.16	5.75	0.65
Ca dissolved**			4.23	6.02	–	6.07	3.59	–
α-K-G mg/100 ml			360	trace	–	1340	145	trace
Pyruvic acid			28	20	–	–	–	–

α-K-G means α-ketoglutaric acid.
* N/10 $Na_2S_2O_3$ ml per ml broth. By LEHMANN-SCHOORL method.
** mg per ml broth.
Initial substrate concentration: glucose 4.51%, gluconate 5.48%, 2-ketogluconate 2.00%.

7 days' incubation at 30°C.

may also proceed through glucose ⟶ gluconate ⟶ 2-ketogluconate ⇢ pyruvate ⇢ α-ketoglutarate, as has been reported by Koepsell[14] in *Pseudomonas fluorescens*. Further support for this presumed pathway is derived from the results of the following experiment.

Formation of α-Ketoglutarate and Pyruvate from Glucose, Gluconate and 2-Ketogluconate.

As shown in Table 18, it was found that both keto acids are formed from a substrate of either Ca-gluconate or glucose and most probably, from K-2-ketogluconate as well. The fact that α-ketoglutaric acid is formed from K-2-ketogluconate was supported by paper chromatography of the fermented liquor. The existence of pyruvic acid was not detected, even by paper chromatography, but this might be due to the fact that the acid is formed early and disappears during prolonged fermentation.

We have no exact evidence for the formation of α-ketoglutarate directly from pyruvate; however, its formation from lactate was observed in another investigation. Thus we feel that α-ketoglutarate formation would probably continue after the disappearance of pyruvate.

V. α-Ketoglutaric Acid Fermentation by a New Bacterium Isolated from Soil.

From the results of the previous experiments the possibility of a wide distribution of α-ketoglutaric acid accumulation among aerobic and facultative anaerobic bacteria was strongly demonstrated. The next investigation to be discribed concerns experiments on a new bacterium, its morphological and physiological properties and its α-ketoglutaric acid fermentation. In this bacterium we found a high yield of α-ketoglutaric acid, about 60% for glucose supplied, even at a 10% glucose concentration.

Information on the classification of this interesting bacterium will be given later in this journal.

Description of the Properties of Bacterium strain No. 84C.

Bacterium strain No. 84C (temporary name) was isolated in 1954 from uncultivated soil in the Tokyo district.

Cells are straight rods with rounded ends, 0.7 to 1.0, by 1.0 to 2.0 microns. Usually single, occasionally in pairs. Longer filamentous irregular forms of 10 to 13 microns are often seen. No capsules or endospores observed. Motile, with a single or occasionally two or more polar flagella. Gram-negative.

Gelatin colonies: small, punctiform (2~3 mm diam.) grayish-white, smooth, moist, glistening, flat. Transparent, becoming opaque. Edge is entire to undulate. No liquefaction.

Gelatin stab: Uniform surface growth. Line of puncture is filiform. No liquefaction.

Agar colonies: Small (as in gelatin colonies) to large, incomplete, circular colonies (6~9 mm diam.). Others are nearly the same as gelatin colonies.

Agar slant: Abundant growth. White, filiform to spreading, glistening. Medium unchanged. Non chromogenic.
Broth: No pellicle. Moderate turbidity with compact, gray sediment. Amount of sediment is moderate.
Litmus milk: Red with acid production. Coagulum is formed. No peptonization.
Potato: Abundant growth. Grayish, moist.
Indole formed.
Nitrates reduced to nitrites.
Ammonia produced.
Starch not hydrolyzed.
H_2S produced.
Methyl red test positive.
Voges-Proskauer test negative.
Citrate utilized.
α-Ketoglutarate utilized.
Uric acid not utilized.
Catalase positive.
Urease negative.
Acid and gas from arabinose, xylose, rhamnose, glucose, fructose, galactose, mannose, lactose, sucrose, maltose, trehalose, melibiose, cellobiose, raffinose, glycerol, mannitol, esculin and salicin.
Some gas from dextrin.
No acid or gas from melezitose, inulin, starch, glycogen, erythritol, sorbitol and dulcitol.
Ratio of H_2 to CO_2 produced is 2.4 in 72 hours' culture in 5% glucose solution (measured by Smith's fermentation tube). The total volume of gas is 4.10 ml.
Fecal to fragrant odor produced in shaken culture.
Anaerobic, facultative.
Temperature relations: optimum 30°C. No growth at 42°C.
Death point: 60°C for 10 minutes.
pH range: optimum pH 7.0 to 8.0. No growth at pH 3.0 or 10.0.
Chromogenesis: Usually grayish-white to light yellowish gray white on ordinary media. No remarkable pigment produced. In shaken culture, yellow to dark yellow pigment and feeble fluorescence occurred.
Source: From uncultivated soil.
Notes: The existence of polar flagella was observed with the electron microscope and by staining method. Temperature relations and pH range were examined in peptone-broth medium.

On α-Ketoglutaric Acid Production by Bacterium strain No. 84C.

Since preliminary experiments have revealed that the α-ketoglutaric acid production of this new strain was over 40% for glucose supplied in 5%

glucose solution, it was considered most important from the point of industrial application to examine in detail the conditions for the accumulation of this acid. The presented experiments relate to this problem.

The Influence of Nitrogen Sources and Other Factors.

In this experiment the basal medium was composed of 7.7 g glucose, 0.1 g KH_2PO_4, 0.04 g $MgSO_4 \cdot 7H_2O$, 0.3 g NaCl, and 10 ppm $FeSO_4 \cdot 7H_2O$ in 100 ml of distilled water. Nitrogen sources were added in quantities so as to give approximately 0.04% N in the medium, which concentration was found to be optimal for α-ketoglutaric acid accumulation in the previous experiment with *Serratia marcescens* No. 18. Sterile $CaCO_3$ (4.0%) was added separately prior to incubation. Fifty ml of sterilized medium in 500 ml flasks were inoculated with a suspension of cells from a bouillon agar slant and incubated for 118 hours at 30°C. on a reciprocating shaker with a rate of 116 r.p.m. After this time the culture fluid was filtered off and the filtrate was subjected to quantitative analysis for α-ketoglutaric acid. The determination was carried out by the polarographic method proposed by Palmer and Jensen[23] instead of by Shimizu's modified Friedmann-Haugen photometric method, because the former was more rapid and its accuracy was found to be identical with the photometric method. The results are summarized in Table 19. As shown in the table it is obvious

Table 19. The influence of nitrogen sources on α-ketoglutaric acid production by *Bacterium* strain No. 84C.

No.	N sources	Concentration (%)	pH		Residual glucose		α-K-G yield	
			Initial	End	g/dl	%	g/dl	%
1	$NH_4H_2PO_4$ + $(NH_4)_2SO_4$	0.12 + 0.12	5.8	5.7	0.69	8.9	3.84	49.7
2	$(NH_4)_2SO_4$	0.19	6.2	5.6	0.38	4.9	3.76	48.6
3	NH_4NO_3	0.11	6.0	5.6	0.57	7.4	3.44	44.5
4	$NH_4H_2PO_4$	0.33	5.8	5.7	0.79	10.2	3.44	44.5
5	$(NH_4)_2HPO_4$	0.19	5.8	5.8	2.08	26.9	2.54	32.9
6	NH_4Cl	0.15	5.8	5.6	0.88	11.4	3.44	44.5
7	Urea	0.09	6.4	5.6	1.07	13.8	3.36	43.4
8	Corn steep liquor	1.00	5.6	5.3	3.90	50.5	0.60	7.8
9	Polypeptone	0.32	5.8	5.4	3.13	40.5	1.30	16.8

N content: corresponding to approximately 0.04% N in the medim.

that this new strain is of value in obtaining a high accumulation of α-ketoglutaric acid. It is also seen that organic nitrogen sources, such as corn steep liquor and polypeptone (Takeda), were found to be unsuitable for acid accumulation, whereas inorganic nitrogen sources, such as ammonium salts, were very effective, especially when $(NH_4)_2SO_4$ or a mixture of

$(NH_4)_2SO_4$ and $NH_4H_2PO_4$ were used. Urea was found to be a good nitrogen source. The highest yield obtained in this experiment was 49.7% of α-ketoglutaric acid for the initial glucose supplied.

Subsequent experiments were conducted to examine constitutive changes in the fermentation medium, as, for example, the combination of nitrogen sources and the additive effects of corn steep liquor and thiamin as trace stimulants. For this purpose, experiments were carried out under the conditions described above, except for the initial glucose concentration and the periodic determination of α-ketoglutaric acid during fermentation. Three cultures were provided for each series.

Table 20. The influence of the concentration of nitrogen sources and the additive effect of thiamin on α-ketoglutaric acid production.

No.	Initial		After 63 hrs				After 88 hrs				After 112 hrs			
	Glucose conc. (%)	pH	pH	R.G. g/dl	R.G. %	α-K-G yield g/dl	α-K-G yield %	pH	R.G. g/dl	R.G. %	α-K-G yield g/dl	α-K-G yield %	pH	R.G. g/dl	R.G. %	α-K-G yield g/dl	α-K-G yield %
1	4.90	5.8	5.8	1.87	38.2	1.64	33.5	5.8	0.73	14.9	2.45	50.0	5.8	0.20	4.1	2.85	58.2
2	5.09	6.0	5.6	1.02	20.0	2.32	45.6	5.8	0.18	3.5	2.90	57.0	5.8	0.07	1.4	2.98	58.6
3	4.99	6.0	5.8	1.72	34.5	1.62	32.5	5.8	0.66	13.2	2.55	51.2	5.8	0.16	3.2	2.95	59.2
4	5.12	5.8	5.4	2.18	42.6	1.68	32.8	5.2	1.52	29.7	2.08	40.6	5.2	0.59	11.5	2.25	43.9
5	5.03	6.0	5.8	2.52	50.0	1.24	24.6	5.8	1.94	38.6	1.55	30.8	5.8	1.05	20.9	1.70	33.8
6	5.14	6.0	5.8	2.30	44.8	1.06	20.6	5.8	2.37	46.1	1.16	22.6	5.8	1.67	32.5	1.60	31.2
7	4.90	6.0	5.8	1.59	32.4	1.78	36.4	5.8	0.52	10.6	2.70	55.1	5.8	0.26	5.3	2.68	54.7

R. G. means residual glucose.

No.	$NH_4H_2PO_4$	$(NH_4)_2SO_4$	NH_4NO_3	Corn steep liq.	KH_2PO_4	K_2HPO_4	Thiamin
1	0.12%	0.12%	—	—	—	0.10%	1 mg/L
2	0.12	—	0.08%	—	—	0.10	〃
3	0.12	0.12	—	—	0.10%	—	〃
4	0.06	0.06	—	0.10%	—	0.10	〃
5	0.06	0.06	—	0.05	—	0.10	〃
6	0.06	0.06	—	0.01	—	0.10	〃
7	0.12	0.12	—	—	—	0.10	—

This experiment (Table 20) showed that rapid fermentation and a high accumulation of α-ketoglutaric acid occured when a mixture of $NH_4H_2PO_4$ and NH_4NO_3, or $NH_4H_2PO_4$ and $(NH_4)_2SO_4$, were used in the quantities quoted in the table.

The N concentration in these experiments was estimated at approximately 0.05% which is within the optimal range for α-ketoglutaric acid fermentation in *Serratia marcescens* No. 18. When the N concentration was reduced to half of this quantity, viz., 0.025%, fermentation was depressed

to a remarkable extent and the accumulation of α-ketoglutaric acid was very low as shown in series No. 4, 5 and 6.

In these experiments, the addition of corn steep liquor did not stimulate fermentation, as it had been observed to do in the previous experiment. The additive effect of thiamin was almost nil, as shown in the comparative experiments of series No. 1 and 7. The highest yield of α-ketoglutaric acid was obtained when 0.12% of both $NH_4H_2PO_4$ and $(NH_4)_2SO_4$ were added to the medium; this yield was 59% of the acid for initial glucose supplied, even though the concentration of glucose was relatively low (approximately 5%). With regard to the effect of iron, we found some stimulation when 1–5 ppm of $FeSO_4 \cdot 7H_2O$ were added, although the effect was not as remarkable as had been observed in *Serratia marcescens*. No. 18. Therefore, subsequent experiments were carried out either with or without the addition of these amounts of ferrous sulfate.

The Influence of Glucose Concentration on α-Ketoglutaric Acid Production.

This experiment was undertaken to study the influence of glucose concentration upon α-ketoglutaric acid fermentation. Glucose was added to the fermentation medium in amounts of 2.5, 5.0, 7.4, 10.0, 12.4, and 16.0%. Basal medium was composed of 0.12 g $NH_4H_2PO_4$, 0.12 g $(NH)_2SO_4$, 0.1 g KH_2PO_4, 0.3 g NaCl, 0.04 g $MgSO_4 \cdot 7H_2O$, and 4 g $CaCO_3$ in 100 ml

Table 21. The influence of glucose concentration on α-ketoglutaric acid production.

No.	Initial glucose concentration (%)	45 Hrs					63 Hrs					87 Hrs				
		pH	R.G.		α-K-G yield		pH	R.G.		α-K-G yield		pH	R.G.		α-K-G yield	
			g/dl	%	g/dl	%		g/dl	%	g/dl	%		g/dl	%	g/dl	%
1	2.56	5.8	0.13	5.1	1.03	40.3	5.9	0.11	4.3	0.99	38.7	6.0	0.06	2.3	0.92	35.9
2	5.05	—	—	—	—	—	5.8	0.96	19.0	1.91	37.8	5.8	0.29	5.8	2.56	50.7
3	7.39	—	—	—	—	—	5.7	2.89	39.1	2.10	28.4	—	—	—	—	—
4	10.10	—	—	—	—	—	5.7	4.83	47.8	1.76	17.4	—	—	—	—	—
5	12.38	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6	16.20	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

No.	111 Hrs					135 Hrs					162 Hrs				
	pH	R.G.		α-K-G yield		pH	R.G.		α-K-G yield		pH	R.G.		α-K-G yield	
		g/dl	%	g/dl	%		g/dl	%	g/dl	%		g/dl	%	g/dl	%
1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	5.8	0.13	2.6	2.64	52.3	5.9	0.13	2.6	2.57	50.9	—	—	—	—	—
3	5.7	0.72	9.7	3.86	52.2	5.9	0.22	3.0	4.12	55.8	—	—	—	—	—
4	5.6	2.53	25.0	4.06	40.2	—	—	—	—	—	5.2	1.72	17.0	4.36	43.2
5	5.8	5.32	43.0	1.15	9.3	—	—	—	—	—	5.8	4.51	36.5	1.30	10.5
6	5.8	8.10	50.0	0.57	3.5	—	—	—	—	—	5.8	7.32	45.2	0.46	2.8

of distilled water. The initial pH was determined as 6.0. The experimental and analytical method were identical with the former experiment. It may be seen (Table 21, Fig. 11) that the lower the concentration of glucose, the more rapid the fermentation, and the optimum concentration was found to be approximately 5% to 7%. In concentrations higher than 10% fermentation was depressed remarkably and it continued in a weak and prolonged manner for more than 162 hours. In this case the residual glucose was estimated to be very high. Although an inhibition resulted from the high concentration of the substrate, it was thought that fermentation might be able to proceed in the presence of relatively high concentrations of glucose provided that successive or intermittent feeding of the substrate was carried on. Experiments relating to this problem, and some good yields of the acid obtained in relatively high concentrations of glucose, without special feeding methods will be described in the subsequent sections.

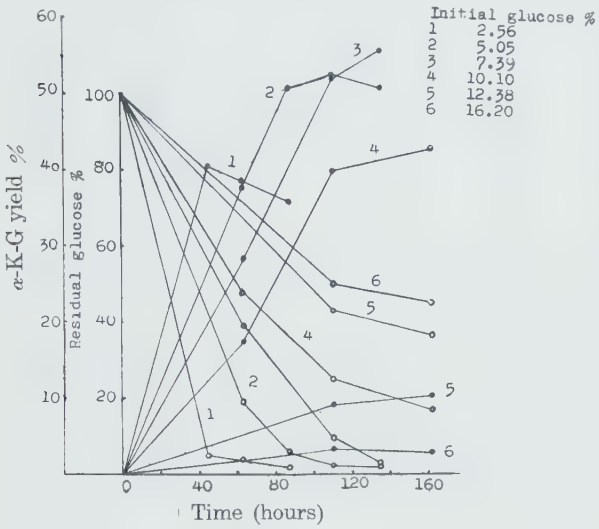


Fig. 11. The influence of glucose concentration on α-ketoglutaric acid production.

The Influence of Arsenite and NaCl on α-Ketoglutaric Acid Production.

Since we had data showing that arsenite and NaCl as additive constitu-

Table 22. The influence of arsenite on α-ketoglutaric acid production.

No.	$NaAsO_2$	pH		Residual glucose		α-Ketoglutaric acid yield	
	mol	Initial	End	g/dl	%	g/dl	%
1	—	5.8	5.9	0.10	2.0	2.35	47.2
2	1×10^{-5}			0.13	2.6	2.41	48.4
3	1×10^{-4}			0.14	2.8	2.35	47.2
4	5×10^{-4}			0.14	2.8	2.32	46.0
5	1×10^{-3}		5.6	0.16	3.2	2.19	44.0
6	5×10^{-3}		8.4	3.90	78.3	—	—

Medium: glucose 4.98%, $NH_4H_2PO_4$ 0.12%, NH_4NO_3 0.08%, KH_2PO_4 0.1%, $MgSO_4\cdot7H_2O$ 0.04%, $FeSO_4\cdot7H_2O$ 5 ppm, $CaCO_3$ 4.0%.
Incubated for 90 hours.

Table 23. The influence of NaCl on α-ketoglutaric acid production.

No.	NaCl	pH		Residual glucose		α-Ketoglutaric acid yield	
	%	Initial	End	g/dl	%	g/dl	%
1	—	5.8	5.9	0.10	2.0	2.35	47.2
2	0.3	6.0	5.8	0.10	2.0	2.35	47.2
3	0.5	6.0	5.6	0.26	5.2	2.28	45.8
4	1.0	6.0	5.2	0.85	17.1	1.90	38.2

Medium: same as above.
Incubated for 90 hours.

ents stimulated α-ketoglutaric acid fermentation in *Serratia marcescens* No. 18, it seemed wise to try to confirm these results in this new strain, which is quite different from *Serratia marcescens*.

The results of the experiments (Tables 22 and 23), contrary to the above facts, quite argue against the stimulative effects of these additive constituents in this bacterium. It is not clear, however, whether this is due to a difference in the pathways of glucose catabolism or due to other differences between the strains. Therefore, subsequent experiments were set up in which no NaCl or 0.3% NaCl was added, which was thought to be within the limits of tolerance.

Periodical Determination of α-Ketoglutaric Acid During Fermentation.

In order to follow the stepwise formation of α-ketoglutaric acid during fermentation, a culture of 10 L of medium containing 4.67% glucose with basal components was grown in a glass jar fermenter provided with a stirrer. Sterile air was introduced at the rate of 1.25 vol. per volume of the medium per minute. The stirring rate was 300 r.p.m. and the incubation temperature was maintained at 30°C.±1°.

The determination of α-ketoglutaric acid was carried out by the photometric method. From the results (Table 24), it is evident that the yield of α-ketoglutaric acid during each stage of fermentation always exceeded 0.5 mole per mole of glucose consumed. A similar phenomenon was reported by Koepsell, Stodola and Sharpe[2] in *Pseudomonas fluorescens* NRRL B-6, although they did not obtain a yield as high as the 0.9 mole of acid per mole of glucose that we obtained.

This seemed very interesting, not only from the point of industrial production of α-ketoglutaric acid, but also with regard to the mechanism of α-ketoglutaric acid fermentation. Provided the oxidative reaction proceeds via the Warburg-Lipmann-Dickens hexosemonophosphate shunt which involves the oxidation of glucose-6-phosphate via 6-phosphogluconate and 6-phospho-2-ketogluconate to 5-phosphopentose, with continued successive 1-carbon degradations of the carbohydrate molecule until one mole of triose-phosphate plus three moles of CO_2 have been produced, the predicted theoretical yield of α-ketoglutaric acid will be 0.5 mole per mole of glucose consumed.

Table 24. Results of α-ketoglutaric acid production by an experimental jar fermenter.

Hours	pH	Residual glucose	α-Ketoglutaric acid yield		
		%	g/dl	%	mole
12	5.9	77.0	0.48	10.3	0.60
24	5.9	67.1	0.87	18.6	0.77
36	5.9	57.1	1.28	27.4	0.86
48	5.9	44.6	1.63	34.9	0.86
60	5.9	34.5	1.88	40.2	0.83
72	5.9	23.3	2.13	45.6	0.80
84	5.9	21.8	2.31	49.4	0.86
90	5.9	19.8	2.42	51.8	0.87
96	5.9	18.9	2.52	53.9	0.90
108	5.9	14.2	2.58	55.2	0.87
120	5.9	7.3	2.62	56.1	0.82
132	6.2	0.53	2.44	52.2	0.70

Medium: glucose 4.67%, $(NH_4)_2SO_4$ 0.12%, $NH_4H_2PO_4$ 0.12%, KH_2PO_4 0.1%, NaCl 0.3%, $MgSO_4 \cdot 7H_2O$ 0.04%, $FeSO_4 \cdot 7H_2O$ 1 ppm, $CaCO_3$ 4.0%.
Initial pH: 6.2.

Since the results of the present experiment did not fit this version of the oxidative pathway, we must assume that the main route of oxidation proceeds in another ways, and it seems most probable that the reaction, involves either the Emden-Meyerhof scheme or phosphogluconate $\longrightarrow C_5 \longrightarrow C_3 + C_2 \longrightarrow$ α-ketoglutarate, or else the reaction of $C_6 \longrightarrow C_5 \longrightarrow$ directly to α-ketoglutarate; in these oxidations the theoretical yields of α-ketoglutaric acid always exceed 0.5 mole per mole of glucose. The last reaction is more probable in consideration of the recent research of Weinberg and Doudoroff[24], who followed α-ketoglutaric acid formation almost quantitatively, by the presumed route of the transformation of L-arabinose $\longrightarrow$ L-arabinolactone $\longrightarrow$ L-arabonic acid $\longrightarrow$ α-ketoglutaric acid, in resting cells of *Pseudomonas saccharophila*.

On α-Ketoglutaric Acid Fermentation by Feeding Methods.

By adding the substrate continuously or semicontinuously during fermentation while transformation of the substrate to the product is taking place, the total concentration of the substrate in the fermenter is enhanced to a certain extent, resulting in a high efficiency of production provided that the microorganisms are tolerant physiologically to the resulting environmental conditions.

For this purpose and by reason of its slow fermentation in relatively high concentrations of glucose, *Bacterium strain No. 84C* was used. After a preliminary incubation for 45 hours in 5% glucose solution, growth and fermentation activities can still be considered vigorous, glucose was added

in an amount to raise the total glucose to approximately 10%. Notwithstanding the small scale of laboratory flask fermentation, and the fact that only one step of feeding was done during the fermentation, the results of this experiment support the suggested advantages of feeding for α-ketoglutaric acid production.

The experimental procedure was as follows. Basal medium identical with that of the previous experiment, on the influence of glucose concentration, was used. To 100 ml lots of the medium, 5 g of glucose and separately sterilized $CaCO_3$, in the amount of 4.0%, were added prior to incubation. Fifty ml of medium in 500 ml flasks was inoculated with a suspension of cells grown on bouillon agar slants and incubated at 30°C. on a reciprocating shaker with a rate of 116 r.p.m. The initial pH was 5.6. Eight identical flask cultures were grown in order to permit analysis of the fermented liquor at intervals. After 45 hours' incubation, when the glucose was reduced to about 30% of the initial content, 10 ml of 35% glucose solution plus 2.8 g of sterile $CaCO_3$ were fed in experiment series No. 1, and 10 ml of 35% glucose solution containing 45 mg of $NH_4H_2PO_4$, plus 2.8 g of sterile $CaCO_3$ were fed in experiment series No. 2; thereafter fermentation was allowed to proceed. As shown in Table 25, the control experiment (experiment series No. 3), started at a glucose concentration of 10.1%, had a very weak and prolonged fermentation. A considerable quantity of glucose remained even after 162 hours' incubation and at that time the yield of α-ketoglutaric acid was about 80% of the yield obtained by feeding in experiment series No. 2. The best results were obtained in experiment series No. 2 which was supplemented with both glucose and nitrogen sources. A

Table 25. The effect of feeding on α-ketoglutaric acid production. (I)

No.	Initial glucose concentration	45 Hours (just prior to feeding)					87 Hours					111 Hours				
		pH	R.G.		α-K-G yield		pH	R.G.		α-K-G yield		pH	R.G.		α-K-G yield	
	%		g/dl	%	g/dl	%		g/dl	%	g/dl	%		g/dl	%	g/dl	%
1	5.05	5.6	1.56	30.9	1.42	28.1	5.8	4.55	45.6	1.99	19.9	5.7	4.31	43.2	2.33	23.4
2	5.05	5.6	1.56	30.9	1.42	28.1	5.8	4.10	41.1	2.37	23.7	5.7	3.26	32.7	2.98	29.9
3	10.10	—	—	—	—	—	—	—	—	—	—	5.6	2.53	25.0	4.06	40.2

No.	135 Hours					162 Hours					186 Hours					Total glucose concentration
	pH	R.G.		α-K-G yield		pH	R.G.		α-K-G yield		pH	R.G.		α-K-G yield		
		g/dl	%	g/dl	%		g/dl	%	g/dl	%		g/dl	%	g/dl	%	%
1	—	—	—	—	—	5.7	3.50	35.1	2.83	28.4	5.7	2.99	30.0	2.98	29.9	9.98
2	5.7	2.08	20.8	3.90	39.0	5.6	1.13	11.3	4.67	46.8	5.8	0.29	2.9	5.51	55.2	9.98
3	—	—	—	—	—	5.2	1.72	17.0	3.82	37.8	—	—	—	—	—	10.10 without feeding

yield of 46.8% of α-ketoglutaric acid to total glucose supplied was attained after 162 hours' incubation, and this reached 55.2% after 186 hours' incubation. The inferior yield of experiment series No. 1 might be considered to be due to the lack of a nitrogen source for complete fermentation. The stimulus of feeding was also observed in the following experiments in which fermentation was started in a 7.7% glucose solution and after 94 hours glucose and a nitrogen source, plus $CaCO_3$, were fed until the total glucose reached 12.3%. The results are given in Table 26 and Fig. 12. In the highest yield with experiment series No. 1, α-ketoglutaric acid production reached 48.7% for total glucose supplied after 238 hours' incubation. The control experiment with 12.3% total glucose and no feeding, resulted in an acid yield of only 10.5%, even after 166 hours' incubation, while at that time the feeding experiments showed 33.6%, 37.9% and 44.2% acid, respectively.

Table 26. The effect of feeding on α-ketoglutaric acid production. (II)

No.	Initial glucose concentration	94 Hoors (just prior to feeding)					111 Hours					142 Hours				
		pH	R.G.		α-K-G yield		pH	R.G.		α-K-G yield		pH	R.G.		α-K-G yield	
	%		g/dl	%	g/dl	%		g/dl	%	g/dl	%		g/dl	%	g/dl	%
1	7.73	5.7	1.39	18.0	2.92	37.8	—	—	—	—	—	5.6	2.53	20.5	4.32	35.0
2	7.73	5.7	1.39	18.0	2.92	37.8	—	—	—	—	—	5.6	3.44	27.9	3.60	29.2
3	7.73	5.7	1.39	18.0	2.92	37.8	—	—	—	—	—	5.6	1.69	13.7	4.44	36.0
4	12.38	—	—	—	—	—	5.8	5.32	43.0	1.15	9.5	—	—	—	—	—

No.	166 Hours					190 Hours					238 Hours					Total glucose concentration
	pH	R.G.		α-K-G yield		pH	R.G.		α-K-G yield		pH	R.G.		α-K-G yield		
		g/dl	%	g/dl	%		g/dl	%	g/dl	%		g/dl	%	g/dl	%	%
1	5.8	1.85	15.0	4.67	37.9	5.6	0.97	7.9	5.40	43.8	6.2	0.26	2.1	6.00	48.7	12.33
2	5.8	2.43	19.7	4.14	33.6	5.7	1.13	9.2	4.68	37.9	5.9	0.22	1.8	4.44	36.0	12.33
3	5.8	0.29	2.4	5.45	44.2	7.0	0.13	1.1	5.52	44.8	7.0	0.13	1.1	5.70	46.2	12.33
4	5.8	4.51	36.5	1.30	10.5	—	—	—	—	—	—	—	—	—	—	12.38 without feeding

Basal medium: $NH_4H_2PO_4$ 0.12%, $(NH_4)_2SO_4$ 0.12%, KH_2PO_4 0.10%, $MgSO_4 \cdot 7H_2O$ 0.04%, NaCl 0.30%, $CaCO_3$ 4.0%.

Initial pH: 6.0.

No. 1: fed with 10 ml 35.3% glucose solution containing 50 mg $NH_4H_2PO_4$ and $(NH_4)_2SO_4$ mixture plus 3 g sterile $CaCO_3$.

No. 2: fed with 10 ml 35.3% glucose solution containing 50 mg $NH_4H_2PO_4$, 50 mg $(NH_4)_2SO_4$, 10 mg KH_2PO_4, 4 mg $MgSO_4 \cdot 7H_2O$ and 30 mg NaCl, plus 3 g sterile $CaCO_3$.

No. 3: fed with 10 ml 35.3% glucose solution containing 50 mg $NH_4H_2PO_4$ and NH_4NO_3 mixture plus 3 g sterile $CaCO_3$.

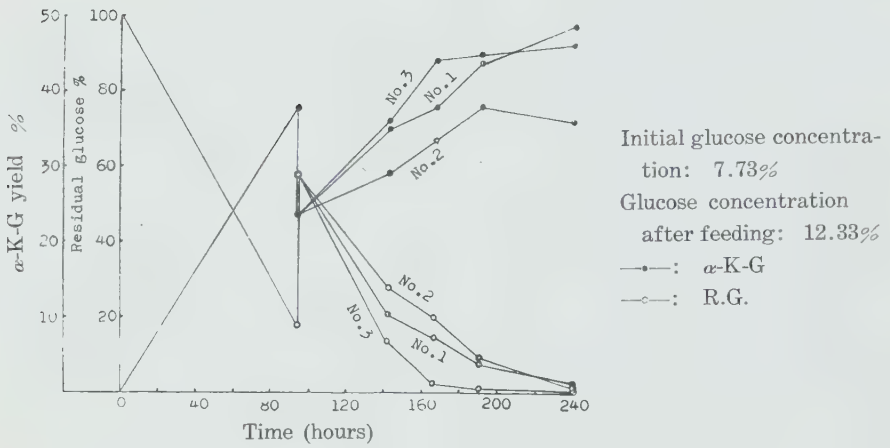


Fig. 12. The effect of feeding on α-ketoglutaric acid production. (II)

Maximum Yields of α-Ketoglutaric Acid Obtained.

From the standpoint of α-ketoglutaric acid production it is obvious that *Bacterium strain No. 84C* has an excellent possibility for industrial application; however, in high concentrations of glucose, the time needed for complete fermentation was noticeably prolonged, even when feeding of the substrate was adopted. From several experiments, carried out to push the yield as high as possible without feeding, we obtained some successful results, as snown in Table 27 and Fig. 13. The quantity of added $CaCO_3$, as well as the basal medium components, were found to affect α-ketoglutaric acid production. The medium used in this experiment was rather rich in

Table 27. α-Ketoglutaric acid production in 10.4% glucose concentration without feeding.

Hours	pH	Residual glucose %	α-Ketoglutaric acid yield %
0	6.6	—	—
12	6.5	77.6	7.4
24	5.8	59.8	16.6
36	6.0	45.0	28.5
48	6.3	29.2	40.7
60	6.0	16.6	51.4
72	8.1	8.1	56.3

Note: The appearance of pyruvate was only detected before 36 hours' incubation by paper chromatography of its 2, 4-dinitropnenylhydrazone.

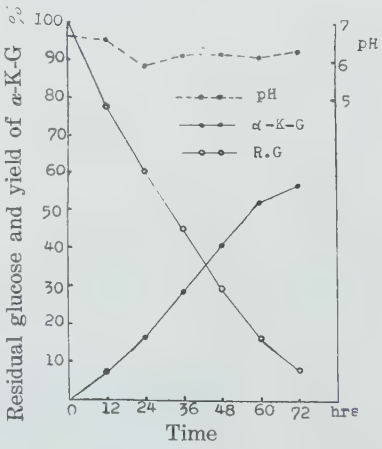


Fig. 13. α-Ketoglutaric acid production in 10.4% glucose concentration without feeding.

nitrogen sources and in the quantity of added $CaCO_3$; it contained 0.135 g $(NH_4)_2SO_4$, 0.135 g $NH_4H_2PO_4$, 0.04 g $MgSO_4 \cdot 7H_2O$, 0.1 g KH_2PO_4 and 2 ppm $FeSO_4 \cdot 7H_2O$ in 100 ml of distilled water. Eight grams of sterile $CaCO_3$ were added separately prior to incubation. NaCl was not added.

The incubation procedure was identical with that of the previous experiments. The determination of α-ketoglutaric acid was made by the photometric method and almost all of the acid was actually recovered from the fermented liquor. As shown, an acid yield of 56% was attained after three days' fermentation, even in media with a 10% concentration of glucose.

These excellent yields in the presence of relatively high concentrations of glucose strongly indicate that this new strain is of high industrial value for α-ketoglutaric acid production.

The Production of α-Ketoglutaric Acid from Various Carbohydrates.

In this experiment fructose, maltose, galactose and sucrose, as well as glucose, were tested for acid production and the acid hydrolysate of starch was also examined as a substrate. The result are summarized in Table 28.

Table 28. α-Ketoglutaric acid production from various carbohydrates.

Carbohydrates	Concentration	After 65 hours				After 89 hours			
		pH	R.G.		α-K-G yield		pH	R.G.		α-K-G yield	
	%		g/dl	%	g/dl	%		g/dl	%	g/dl	%
D-Glucose	5.15	5.4	0.98	19.0	2.21	42.9	5.6	0.25	4.9	2.77	53.8
D-Galactose	5.09	5.6	1.86	36.5	0.80	15.7	5.6	1.67	32.8	0.84	16.5
D-Fructose	4.93	5.4	0.91	18.5	2.11	42.8	5.8	0.16	3.2	2.81	57.0
Sucrose	4.63	5.4	2.59	56.0	0.51	11.0	5.6	2.50	54.0	0.57	12.3
Maltose	4.74	6.0	1.72	36.3	0.54	11.4	5.8	1.42	30.0	0.54	11.3
Starch hydrolysate	5.29 as glucose	5.1	1.05	19.8	1.95	36.8	5.1	0.36	6.8	2.43	46.0

Basal medium: $NH_4H_2PO_4$ 0.12%, $(NH_4)_2SO_4$ 0.12%, KH_2PO_4 0.10%, $MgSO_4 \cdot 7H_2O$ 0.04%, NaCl 0.30%, $FeSO_4 \cdot 7H_2O$ 1 ppm, $CaCO_3$ 4.0%.

The high yield were obtained in glucose-and fructose-containing media, each corresponding to 53.8% and 57.0% of α-ketoglutaric acid for sugars supplied. The acid hydrolysate of starch was proved to be suitable for α-ketoglutaric acid production, the yield reaching 46% for the contained reducing sugar.

The other sugars, i.e., galactose, maltose and sucrose, although they were metabolized into α-ketoglutaric acid to some extent, gave very low rates of fermentation and assimilation.

DISCUSSION

The main object of this investigation was to find microorganisms which possess a marked activity in α-ketoglutaric acid production. To date, ex-

cept for the researches of Lockwood *et al.*[1], Koepsell[14], Koepsell, Stodola and Sharpe[2], Katagiri[4], Masuo and Wakizaka[5], we have found no publications on this subject. An interesting fact emerging from the above researches is that the microorganisms capable of producing large amounts of α-ketoglutaric acid are generally distributed in the genera *Pseudomonas*, *Escherichia* and *Bacterium* (the last is recognized as a *nomen genericum rejeciendum*). These are thought to metabolize glucose aerobically or anaerobically and are well known as aerobic or facultative anaerobic bacteria. From the point of view of glucose catabolism, *Pseudomonas fluorescens* NRRL B-6, in which α-ketoglutaric acid production has been studied by Koepsell *et al.*, is a typical species, with an oxidative pathway of glucose ⟶ gluconate ⟶ 2-ketogluconate ⇢ pyruvate ⇢ α-ketoglutarate. In many *Escherichia coli* strains a phosphogluconate pathway, besides the Emden-Meyerhof scheme, has been found. These facts strongly suggested the possibility that α-ketoglutaric acid accumulation may take place in various other facultative anaerobic bacteria as well as aerobic bacteria.

Previously it was reported by Ikeda[25] that *Serratia marcescens* No. 18, metabolizes glucose into gluconate and 2-ketogluconate oxidatively; in many strains of *Gluconobacter* and *Acetobacter* as well this oxidative pathway has been demonstrated by a number of investigators. Furthermore, Asai *et al.*[10] reported that *Serratia marcescens* No. 18 is able to change glucose into acetoin to a remarable extent in stationary culture, notwithstanding the fact that the direct oxidative pathway of glucose is followed in shaken cultures. This phenomenon was also observed in stationary cultures of *Bacillus mesentericus*, but the oxidative metabolites of glucose in shaken culture yet have not been examined in this form.

In view of these phenomena, it was considered interesting to ascertain whether such aerobic or facultative anaerobic bacteria as *Bacillus mesentericus* and *Bac. subtilis* or *Bacterium succinicum*[7] which is thought to be analogous to the *Escherichia coli* group, are able to take an oxidative pathway via gluconate and 2-ketogluconate, with further degradation to pyruvate or α-ketogluconate, or whether they are lacking this pathway in shaken cultures. We therefore examined the oxidative metabolic products of these organisms in shaken cultures, and found that α-ketoglutaric acid accumulation occurs in a variety of strains tested.

Summarizing the results, α-ketoglutaric acid accumulation in cultures of *Pseudomonas* strains, including *Pseudomonas fluorescens* NRRL B-6, did not attain such a high level as Koepsell *et al.*[2] reported for their strain of *Pseudomonas fluorescens*. In *Serratia marcescens* No. 18, however, we found a larger accumulation of the acid than was expected. A yield of approximately 25 g of the acid per 100 g of glucose in 7.6% glucose containing medim was obtained with this organism. This finding of α-ketoglutaric acid accumulation in the genus *Serratia* is new, and our expectation of the existence of oxidative pathway of glucose catabolism was fulfilled by this investigation in which we succeeded in isolating gluconate and 2-ketogluco-

nate as intermediary products of glucose oxidation.

Further findings consistent with the above, were that some strains of *Bacillus megatherium*, *Bacillus natto* and *Bacterium succinicum* could accumulate α-ketoglutaric acid during the oxidative fermentation of glucose, although whether gluconate and 2-ketogluconate are the intermediary products has not yet been studied. The last strain was reported by Sakaguchi and Tada[7] to form a remarkable amount of succinic acid from citrate.

The most important finding reported here is the isolation of a bacterium, probably belonging to a new genus, (temporaly called *Bacterium strain No. 84C*—classification to be reported later) and its marked ability to accumulate α-ketoglutaric acid, surpassing all reported microorganisms except *Bacterium α-ketoglutaricum*, formerly isolated by Masuo *et al.*[5]

From experiments on the constituents of the fermentation media and some environmental conditions affecting α-ketoglutaric acid fermentation by *Serratia marcescens* No. 18 and *Bacterium* strain No. 84C, it may be conclued that the optimum concentration of glucose as a substrate, may be raised to 10% in the latter strain, provided that the mixed nitrogen sources of $(NH_4)_2SO_4$ and $NH_4H_2PO_4$ are added in quantities of 0.135% of each, along with approximately 2 ppm of $FeSO_4 \cdot 7H_2O$, an excess of $CaCO_3$ (over 6%), and adequate amounts of KH_2PO_4 and $MgSO_4 \cdot 7H_2O$. The stimulative effect of iron was markedly observed in *Serratia marcescens* No. 18, as had been reported by Koepsell *et al.*, but no effect of thiamin was observed in *Bacterium strain No. 84C* contrary to the situation in *Serratia marcescens* No. 18. NaCl and arsenite were proved to be stimulative for the α-ketoglutaric acid fermentation of *Serratia marcescens* No. 18, while in *Bacterium* strain No. 84C, no effects were observed. The differences between these strains probably lie in their physiological and biochemical properties, but the nature of the difference is not yet understood. We consider it probable that there is a difference in the oxidative pathways of glucose and the related enzyme systems in the two organisms.

This consideration is supported by our observations on chemical changes during fermentation; in the former strain, gluconate and 2-ketogluconate were easily metabolized into α-ketoglutarate, while in the latter the oxygen uptake of gluconate was scarcely apparent and no oxygen uptake of 2-ketogluconate was observed.

Concerning the oxidative catabolism of glucose to α-ketoglutarate, a direct oxidative metabolism of glucose, i.e., glucose ⟶ gluconate ⟶ 2-ketogluconate ⟶ pyruvate ⟶ α-ketoglutarate, was strongly suggested in *Pseudomonas fluorescens* and *Gluconoacetobacter cerinum*, however, pyruvate has not been detected to any extent during the fermentation of glucose in *Serratia marcescens* No. 18. It is interesting that the formation of pyruvate by this organism was only found during the fermentation of fructose.

However, the fact that the initial step of glucose oxidation to gluconate and 2-ketogluconate, and the formation of α-ketoglutarate, have been clearly demonstrated in *Serratia marcescens* No. 18 and in *Gluconoacetobacter*

cerinum, indicates that the oxidative pathways followed by these two organisms are very probably analogous to that of *Pseudomonas fluorescens*.

Bacterium strain No. 84C demands further consideration, however, as it can not metabolize 2-ketogluconate, and metabolizes almost no gluconate oxidatively. The direct oxidative pathway shown above might not be used for glucose catabolism, and the fact that α-ketoglutarate always yields over 0.5 mole per mole of glucose, accounting for approximately 0.9 mole at the maximum, suggests that the Emden-Meyerhof course, or, the course of $C_6 \longrightarrow C_5 \longrightarrow [C_3 + C_2] \longrightarrow$ α-ketoglutarate or of $C_6 \longrightarrow C_5 \longrightarrow$ directly to α-ketoglutarate might be predominant in this organism; by these metabolic routes a yield of up to 0.9 mole of α-ketoglutarate per mole of glucose could theoretically be expected. Another biochemical pathway of glucose, proposed recently by Entner and Doudoroff[26] for *Pseudomonas saccharophila*, involving the conversion of glucose $\longrightarrow$ gluconate $\longrightarrow$ 2-keto-3-desoxy-gluconate $\longrightarrow$ 2 pyruvate, should be also taken into consideration. This pathway has been shown to be followed in phosphorylative transformation.

Concerning the formation of α-ketoglutarate from pyruvate, we have not yet performed the fundamental experiments; however, the presumed pathway of 2 pyruvic acid $\xrightarrow{+O}$ pyruvic acid + acetyl CoA $\xrightarrow{+O}$ α-keto-glutaric acid + CO_2 + H_2O, which has been proposed recently by Katagiri, Tochikura and Imai[4] for growing cells of the *Escherichia coli* group, is thought to be followed, judging by its theoretical yield of α-ketoglutaric acid i.e. one mole of the acid per 2 moles of pyruvic acid arising from one mole of glucose.

SUMMARY

That α-ketoglutaric acid fermentation is widely distributed in micro-organisms was confirmed by experiments with shaken cultures of *Pseudomonas fluorescens*, *Serratia marcescens*, *Bacillus megatherium*, *Bacillus natto* (a variant of *Bacillus subtilis*), *Bacterium succinicum*, *Gluconoacetobacter cerinum* and a new *Bacterium* strain No. 84C (temporary name), isolated from soil by the authors. The maximum yield of α-ketoglutaric acid among the species examined was obtained with *Bacterium* strain No. 84C which produced up to 56% of the acid for total glucose supplied in a 10% glucose concentration after 72 hours' incubation. A description of the morphological and physiological properties of this bacterium is presented.

With regard to the constituents of the fermentation medium, various nitrogen and carbon sources and their optimum concentrations, as well as the additive influences of metals, arsenite and vitamins, were examined, especially in relation to α-ketoglutaric acid fermentation in *Serratia marcescens* and *Bacterium* strain No. 84C, both of which are considered to have value for industrial application. α-Ketoglutaric acid was formed from D-glucose, D-mannose, D-galactose, D-fructose, D-xylose, L-arabinose, L-rham-

nose, K-gluconate and K-2-ketogluconate in *Serratia marcescens* No. 18.

In the case of *Bacterium* strain No. 84C, α-ketoglutaric acid formation was tested with limited carbon sources involving of D-glucose, D-galactose, D-fructose, sucrose, maltose, and Ca-gluconate, and the results indicate that all substrates were utilized for acid formation.

The optimum range of glucose concentration in the medium was found to lie at 3~4% for the former strain; in the latter it was somewhat at a higher, 5~7%, and in this strain complete fermentation was possible even glucose concentration of 10%, provided that the medium was balanced in other components and an excess of $CaCO_3$ was added.

Among inorganic nitrogen sources, ammonium salts such as $(NH_4)_2SO_4$, $NH_4H_2PO_4$, or a mixture of the two, were found to be suitable, and a rather sharp peak seemed to exist in the range of limited concentration, 0.15~0.17% in the case of $(NH_4)_2SO_4$ in medium containing 7% glucose. The highest yield was obtained by using *Bacterium* strain No. 84C and a medium containing 0.135 g $(NH_4)_2SO_4$ and 0.135 g $NH_4H_2PO_4$ with a 10% concentration of glucose.

The addition of iron to the medium increased the acid yield in *Serratia marcescens*; the optimum quantity was found to be 0.1~0.6 ppm Fe.

Remarkably interesting were the effects of adding arsenite and thiamin; these gave in optimum concentrations a marked increase in acid production in *Serratia*, while in *Bacterium* strain No. 84C no stimulative effect was observed.

From the investigation of chemical changes during fermentation and from the isolation of intermediates of the initial stages of fermentation, it may be said that α-ketoglutaric acid formation in *Pseudononas fluorescens* No. 33F, *Serratia marcescens* No. 18 and *Gluconoacetobacter cerinum* most probably proceeds via a direct oxidative pathway as has been proposed by Koepsell *et al.* In *Bacterium* strain No. 84C, however, the Emden-Meyerhof scheme, or some alternative pathway involving a direct oxidation not involving 2-ketogluconate as an intermediate, are considered possible.

POSTCRIPT

This communication includes the results of the following reports which have been published in Japanese, plus the recent investigation of a new α-ketoglutaric acid producing bacterium.

1) On the Production of α-Ketoglutaric and Pyruvic Acids by *Pseudomonas* and *Serratia*.
   Asai, T. and Aida, K.: *J. Agr. Chem. Soc. Japan*, **26**, 528 (1952).
2) On the Production of α-Ketoglutaric Acid by *Bacillus megaterium* and *Bacillus natto*.
   Asai, T., Aida, K. and Sugisaki, Z.: *J. Agr. Chem. Soc. Japan*, **27**, 129 (1953).
3) On α-Ketoglutaric Acid Fermentation. Part 1.
   Asai, T., Aida, K. and Sugisaki, Z.: *J. Agr. Chem. Soc. Japan*, **28**, 479 (1954).

4) On α-Ketoglutaric Acid Fermentation. Part 2.
SUGISAKI, Z., AIDA, K. and ASAI, T.: *J. Agr. Chem.Soc. Japan*, **29**, 296 (1955).

5) The Formation of α-Ketoglutaric and Pyruvic Acids by *Gluconoacetobacter cerinum*.
ASAI, T., SUGISAKI, Z. and AIDA, K.: *J. Agr. Chem. Soc. Japan*, **29**, 300 (1955).

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